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Title of Dissertation: "The Differential Expression of Calcitonin Gene-Related Peptide, α-CGRP mRNA, Choline Acetyltransferase, and Low Affinity Nerve Growth Factor Receptor in Cranial Motoneurons After Hypoglossal Nerve Injury During Postnatal Development"

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ABSTRACT

Title of Dissertation: The Differential Expression of Calcitonin Gene-Related Peptide, α-CGRP mRNA, Choline Acetyltransferase, and Low Affinity Nerve Growth Factor Receptor in Cranial Motoneurons After Hypoglossal Nerve Injury During Postnatal Development

Kristen M. Blake Bruzzini, Doctor of Philosophy, 1996

Dissertation directed by: Rosemary C. Borke, Ph.D.
Associate Professor
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This study examined the temporal expression of calcitonin gene-related peptide (CGRP), choline acetyltransferase (ChAT), and low-affinity nerve growth factor receptor (LNGFR) in hypoglossal motoneurons during postnatal development and after hypoglossal nerve injury. CGRP is a putative neurotrophic factor that coexists with acetylcholine, the neurotransmitter present in hypoglossal motoneurons. ChAT is a key enzyme involved in the synthesis of acetylcholine and is a specific marker for cholinergic neurons. Hypoglossal motoneurons transiently express LNGFR. These molecules were assessed using immunocytochemistry. Ribonuclease protection assay was performed to determine if peptide changes were preceded by changes in α- CGRP mRNA expression. Postnatally, CGRP and ChAT are expressed at low levels during the first postnatal week and increase to maximal levels during the second postnatal

week. While ChAT expression is maintained at these levels in adult motoneurons, CGRP is decreased and maintained at low levels in the adult. LNGFR is expressed at maximal levels during the first postnatal week and disappears by the third postnatal week. The onset of the CGRP, ChAT, and LNGFR changes was the same after axonal damage to motoneurons of increasing postnatal ages. The progression of changes in CGRP after onset was age-related and likely to depend upon the developmental stage of motoneuron interaction with its target and/or its environment. α-CGRP mRNA was upregulated soon after each nerve injury in the 10 and 21 day postnatal (dpn) rats, but subsequent elevations in gene expression were limited to the 21 dpn experimental rats. After onset, the progression of changes in ChAT and LNGFR in postnatal motoneurons was not age-related but injury-specific as reported for adult motoneurons (Armstrong et al., 1991, Borke et al., 1992). The magnitude of the initial reduction of ChAT was injury-specific for the 21 dpn rats but not for the 10 and 14 dpn rats. In all injury paradigms, ChAT returned to normal levels by 40 dpo except in the 10 dpn rats nerve transected rats. The intensity of LNGFR-IR was greater after crush but the response persisted longer after nerve transection. Data for quantitative analysis of neuronal survival and reinnervation suggested that when injury occurs at a postnatal age when the immunoreactive response of CGRP paralleled that of adult motoneurons, neuronal survival could be predicted.

The Differential Expression of Calcitonin Gene-Related Peptide, α-CGRP mRNA, Choline Acetyltransferase, and Low Affinity Nerve Growth Factor Receptor in Cranial Motoneurons After Hypoglossal Nerve Injury During Postnatal Development

by

Kristen M. Blake Bruzzini

Dissertation Submitted to the Faculty of the Department of Anatomy and Cell Biology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1996

DEDICATION

To my loving parents, Donna and Bill for a lifetime of love, patience, and support without which it would have been next to impossible to complete my graduate studies. Mommy and Daddy, thank you for being the first teachers in my life. Your prayers and guidance have led me through many challenges.

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CHAPTER 1.

Introduction

Normal Adult Hypoglossal Nucleus in Rats

The hypoglossal nucleus is located dorsally within the medulla from the level of the pyramidal decussation to the caudal third of the opened fourth ventricle. This nucleus consists of neuronal cell bodies embedded in a neuropil of nerve processes, glial cells, and blood vessels. The hypoglossal nucleus consists mostly of neurons that give rise to axons that innervate the intrinsic and extrinsic musculature of the tongue (Connaughton et al., 1986). In the rat, the neurons of the hypoglossal nucleus range widely in size and shape (10-50 μm). The majority (70%) of the neurons are large (25-50 μm) projection motoneurons to the tongue musculature (Odutola, 1976; Cooper, 1981). The remainder of the neurons are small (10-18 μm) local interneurons localized chiefly in the ventrolateral margin of the nucleus just dorsal to the exiting hypoglossal nerve (Boone & Aldes, 1984a). Hypoglossal motoneurons can vary from quandrangular, to bulbous, to fusiform (Cooper, 1981), whereas interneurons are round to oval in shape (Boone & Aldes, 1984a). Four to five dendritic trunks generally radiate from the cell bodies of the motoneurons and branch into secondary and tertiary branches (Borke et

al., 1988). The dendrites remain within the nucleus on the same side, cross the midline to the opposite nucleus, or extend laterally into the adjacent reticular formation (Cooper, 1981). A single axon usually emerges from one of the proximal dendrites. Axons of motoneurons exit at the ventrolateral border of the nucleus and course through the medulla to emerge from its ventral surface (Boone & Aldes, 1984a).

The hypoglossal nucleus is divided into ventral and dorsal subnuclei (Krammer et al., 1979). The ventral subnucleus is further divided into ventromedial and ventrolateral portions. The ventrolateral portion is small and is located in the caudal third of the hypoglossal nucleus. The ventromedial portion extends throughout the length of the nucleus. The dorsal subnucleus is not subdivided and lies in the rostral two-thirds of the nucleus. The hypoglossal nerve is divided into medial and lateral branches (Krammer et al., 1979). The medial branch of the hypoglossal nerve is formed from axons of motoneurons located in the ventral subnucleus and innervates the geniohyoid (ventrolateral) and genioglossus (ventromedial) muscles. The lateral branch of the nerve arises from motoneurons in the dorsal subnucleus and innervates the styloglossus and hyoglossus muscles. Topographical distribution of hypoglossal motoneurons innervating the intrinsic muscles have not yet been determined (Altschuler et al., 1994). Because the hypoglossal nerve is purely a motor nerve, it is a good model to study peripheral nerve injury. The facial and sciatic nerves are frequently-used models in peripheral

injury studies. However, since these are mixed nerves motoneuronal changes can be influenced by the sensory components of these nerves.

Synaptic inputs to hypoglossal motoneurons originate chiefly from the medullary reticular formation, the spinal V complex, and the nucleus of the solitary tract (Borke et al., 1983). Inputs to the hypoglossal nucleus make synaptic contacts on the somata and the dendrites of the large motoneurons. Four types of synaptic terminals have been identified in the hypoglossal nucleus of the rat: S-, C-, F-/P-, and T- boutons (Borke, 1982; Boone & Aldes, 1984b). Synaptic boutons can be categorized into primarily two categories: spherical and flat/pleomorphic. Spherical boutons are predominantly found along the length of the dendrites. Flat/pleomorphic boutons are generally located proximally on the cell bodies and large dendrites. As in many other systems, the shape of the synaptic vesicle populations correlates with the functional activity of that terminal (Caunaughton et al., 1986; Takasu et al., 1987). Specifically, boutons containing flattened pleomorphic vesicles have been associated with inhibitory effects, and those with spherical vesicles with excitatory effects.

Normal Developing Hypoglossal Nucleus in Rats

During development, the cross sectional area of the nucleus expands 22%. This growth occurs between 7 and 61 days postnatal (dpn), with the majority of the growth taking place between the second and fourth postnatal weeks (Borke, 1982). As the hypoglossal nucleus matures, the cell bodies of the developing neurons in the nucleus undergo changes. Neurons of young rats (7 dpn) contain a slightly eccentric nucleus and an irregularly folded nuclear membrane. The perinuclear cytoplasm is more pronounced, often containing polyribosomes and clusters of rough endoplasmic reticulum (RER) cisterns which are randomly oriented in the periphery of the nucleus (Borke, 1983). As the rat matures, these features become less obvious, and by 21 dpn, the neurons contain a centrally placed nucleus, a regularly contoured nuclear membrane, a less pronounced perinuclear region and parallel lamellae of RER distributed throughout the perikarya (Borke, 1983).

As the young rat develops, the surface of the soma undergoes changes. The surface of neurons in the hypoglossal nucleus at 7 dpn are interrupted by somatic spines that disappear by 21 dpn. These spines contain ribosomes, microfilaments, and smooth-surfaced vesicles (Borke, 1982). They are thought to establish synaptic contact with the surface of the neurons in 7-10 dpn rats. With increasing age the axosomatic boutons appear more frequently and contain more vesicles. By 21 dpn axosomatic boutons constitute the chief structure opposed to the somal surface. S-

boutons containing spherical vesicles are the earliest terminals in contact with the soma at 10 dpn, forming assymetrical axosomatic junctions. F-boutons are not as numerous until the end of the second postnatal week and usually form symmetrical junctions with the neuronal somata. C-boutons, termed subsurface cistern junctions are evident during all postnatal periods. Although these boutons are occasionally invaginated into the soma of motoneurons in 7-13 dpn rats, they are not indented into somata of 21 dpn neurons (Borke, 1982).

Denervation Response of Hypoglossal Motoneurons

Adult Retrograde Reaction

Peripheral axonal injury induces a rapid transformation in the structural and functional state of the neuron. Typically, axotomized neurons undergo dedifferentation and assume a growing rather than a transmitting mode (Matthews & Raisman, 1972). The response of the neurons to peripheral nerve injury is termed the axon reaction. The neuronal appearance seems altered 7-13 days following axotomy (Hall & Borke, 1988). Morphological changes in the cell body of the injured neurons include cell body swelling, nuclear eccentricity and an increase in nucleolar size (Lieberman, 1971; Grafstein & McQuarrie, 1978).

Another important feature of the axon reaction is central chromatolysis. Chromatolysis is characterized by a change in the RER.

Normal RER consists of flat, elongated cisternae arranged in parallel that are lined with membrane attached ribosomes externally, and have free polyribosomes interposed between adjacent cisternae. In neurons which have undergone chromatolysis, the RER is characterized by irregularly organized and shortened cisternal arrays, and detachment of the membrane associated ribosomes (Grafstein & McQuarrie, 1978).

Metabolic changes also occur in the nucleus in response to nerve injury. These changes involve increased RNA synthesis, increased nucleolar RNA, and increased protein content (Watson, 1968) and are due to the shift towards a down-regulation in the synthesis of neurotransmitter-related products and an up-regulation in the synthesis of growth-related products (Lieberman, 1971).

Developmental Retrograde Reaction

Axonal injury to rats of increasing postnatal ages (10 and 21 dpn) produces intrasomatic changes which are similar to those observed in the adult rats after peripheral axotomy. However, major differences exist in the appearance of the retrograde changes and also, the magnitude of the intrasomatic reaction is age dependent. Interestingly, in young rats (10 dpn), the same nuclear reaction is observed in spite of the type of peripheral nerve injury, and it is generally a more exaggerated version of what is normally observed at this time in development (Borke, 1983). The

nuclear reaction continues through the first two postoperative weeks, and then becomes less striking. The first changes in the nucleus are observed within 3-7 days postoperative (dpo). At this time, the nucleus becomes extremely displaced, the nuclear membrane becomes deeply infolded, and free polyribosomes and segments of RER appear to continue to proliferate and form parallel arrays, but by the end of the second post-operative week the cisterns of RER begin to shorten and become disorganized (Borke, 1983).

An interesting capability of immature neurons which is lost in adult neurons is their ability to engulf cell processes (Borke, 1982). It is thought that the purpose of this is to isolate the motoneuron from its surroundings before glial intrusion. Typically, both the S- and C- boutons become engulfed by the somal protrusions of the young injured neurons. Engulfed dendritic processes are frequently observed between 13-40 dpo. In 10 dpn rats, axosomatic contacts and vesicle containing profiles around the soma are engulfed at 3-7 dpo but appear normal in number. This can be compared to the displacement of boutons containing spherical vesicles which is not apparent until 13-20 dpo (Borke, 1982). The immature neurons may not be capable of making the structural changes a mature neuron can make to stabilize synaptic and nonsynaptic attachments displacing surrounding neuronal processes from the neuronal surface (Borke, 1982).

With respect to the perisomatic changes that occur following peripheral nerve injury in the 21 dpn rats, there is again a striking similarity to the responses observed following injury in the adult. Typically, little if any somal engulfment of perisomatic structures is observed in the mature animals (Borke, 1982). The loosening and displacement of the boutons occurs very soon after axotomy and is not dependent on the type of injury (Borke, 1982). Although the reduction in number of axosomatic boutons seems to be of equal magnitude for each type of injury, the boutons appear to return to the somata earlier (13-20 dpo) after crush injury in the 21 dpn rats (Borke, 1982). S- and C-boutons are not frequently seen around the somata by 3-7 dpo after axonal damage to 21 dpn rats (Borke, 1982).

Neuronal Survival

Adult: different types of injury

Motoneurons in mature rats that are separated from their targets undergo cell death but to a lesser degree than is seen in developing or young rats. It is thought that this is due to the fact that as animals mature motoneurons become less dependent on contact with their targets for survival (Snider et al., 1992). Although the age of the rat at the time of the injury is the most crucial factor in determining neuronal survival (Koliatsos et al., 1994) several other factors can also play a role. These

factors include the severity of the nerve injury and whether the axotomized neurons are located in the PNS or CNS. An adult pattern of neuronal survival is generally observed by 21 dpn (Snider & Thanedar, 1989). In adult rats, a hypoglossal nerve crush produces little if any neuronal cell loss and nearly complete reinnervation is established 20 days after nerve crush (Borke, 1982; Borke et al., 1993). When the hypoglossal nerve is transected or resected, however, neuronal cell loss increases and varies depending on the severity of the injury (Snider & Thanedar, 1989; Borke et al., 1993).

Development: contrast to adult

The most important parameter regulating the degree of cell loss after axotomy in mammals is the age at which the axotomy is performed. In young mammals, axotomy and separation of motoneurons from their peripheral targets results in high levels of neuronal cell death compared to adult mammals in which a regenerative response is typical following axotomy (Snider et al., 1992). The age at which axons are injured is also critical to how rapidly the cell loss occurs (Snider et al., 1992). Target dependence at early postnatal ages is a general property of neurons in both the central and peripheral nervous systems of mammals (Snider & Thanedar, 1989). Axotomy in developing neonatal and young rats causes an interruption of the supply of target-derived neuronal growth factors which are thought to play a crucial role in the maturation and survival of

these neurons (Snider et al., 1992). It is also important to note that the age-dependent changes associated with axotomy parallel almost precisely the age-related decreases in dependence of neurons on growth-factors (Snider et al., 1992). Young rats (7 dpn) have been shown to undergo substantial neuronal loss in the nucleus of the injured peripheral nerve. This cell loss is apparent from 9-40 dpo. After nerve crush, by 40 dpo, the number of neurons in the affected nucleus is reduced by 25% compared to the uninjured side following nerve crush (Borke, 1982). Nerve crush in 10 dpn rats does not elicit a significant amount of neuronal loss (13%) until 20 dpo. The loss was considerably less than that of the 7 dpn nerve crush injuries

As mentioned earlier, the 21 dpn rats display little if any cell loss following the crush injury. Their response is similar to that observed in the adult. In the 21 dpn rats, a significant amount of neuronal cell loss (17%) followed only the most severe injury in which the transected ends of the nerve were ligated (Borke, 1982).

Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide synthesized in motoneurons by alternative splicing of mRNA transcribed from the calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983). CGRP occurs in two forms: α-CGRP and β-CGRP. α-CGRP is produced in both the central and peripheral nervous systems and in several endocrine tissues (Amara et al., 1985). β–CGRP is amindated at the COOH-terminal and differs in rats and humans by one and three amino acids respectively (Noguchi et al., 1990). β-CGRP mRNA appears to be expressed in the brain, sensory ganglia, and the thyroid gland in a pattern similar to that seen for α-CGRP mRNA, however the relative expression of each mRNA varies in different cranial nerve nuclei (Amara et al., 1985).

CGRP has been shown to occur throughout the central and peripheral nervous systems. It is in fact considered one of the most abundant peptides in the nervous system with CGRP neurons and fibers distributed widely but unevenly throughout the CNS and PNS (Ishida-Yamamoto & Tohyama, 1989). CGRP is one of the few peptides found in spinal and cranial motoneurons of mammals (Skotfitsch & Jacobowitz, 1985; Kruger et al., 1988).

CGRP is packaged in neuronal cell bodies and conveyed via anterograde axoplasmic transport to neuromuscular junctions of skeletal

muscle (Inaishi et al., 1992; Fernandez & Hodges-Savola, 1994) at a rate of 1 mm/hr (Kashihara et al., 1989). Very little CGRP is transported retrogradely back to the cell body indicating that it is released at the neuromuscular junction (NMJ) (Fernandez & Hodges-Savola, 1994). At the NMJ, CGRP immunoreactivity (CGRP-IR) is confined to the nerve terminal (Takami et al., 1985a) where it is stored in large dense core vesicles (Matteoli et al., 1990). These vesicles represent a small percentage of the total vesicle population in motor nerve terminals (Brewer & Lynch, 1986). Acetylcholine is concentrated in an abundant population of small clear synaptic vesicles (Matteoli et al., 1990). As a result, it is thought that CGRP coexists with acetylcholine in motoneuron nerve terminals and that its release may exert a neuromodulatory or neurotrophic effect at the NMJ (Takami et al., 1985b).

CGRP is thought to be released from axon terminals in a Ca^{++} dependent manner (Uchida et al., 1990). Current information suggests that CGRP binds to specific receptors on the muscle plasma membrane (Popper & Micevych, 1989) where it stimulates acetylcholine receptor (AChR) biosynthesis (Fontaine et al., 1986; New & Mudge, 1986), increases the rate of insertion of AChR into the plasma membrane (New & Mudge, 1986), intensifies nerve-evoked muscle contraction (Takami et al., 1985a) and elevates intracellular cAMP in the target muscles (Laufer & Changeux, 1987). When CGRP is applied externally, it has also been shown to increase the rate of desensitization of AChR (Mulle et al., 1988) and receptor channel conductance (Eusebi et al., 1988). CGRP has also

been shown to enhance the spontaneous release of acetylcholine from the motor end plate (Jinnai et al., 1989). Because specific CGRP binding sites are localized on the postsynaptic membrane at the NMJ, CGRP is believed to exert a direct action on skeletal muscle through a receptor independent from the acetylcholine receptor (Arvidsson et al., 1993).

Ontogeny of CGRP in Cranial Motoneurons

CGRP expression is highly plastic. Appearance of this peptide is believed to correspond to the onset of striated muscle function. During development, the appearance of CGRP occurs in hypoglossal, facial and motor trigeminal nuclei during the late prenatal period when suckling involves lingual, mimetic, and masticatory muscles (Kubota et al., 1988). In the rat hypoglossal nucleus, individual CGRP immunoreactive cells have been observed in late prenatal development at E18/19 (Kubota et al., 1988; Hares & Foster, 1991). CGRP peptide expression increases rapidly during the early postnatal period and then decreases to adult levels by the end of the fourth postnatal week (Kubota et al., 1988; Hares & Foster, 1991).

Response of CGRP to Deafferentation and Denervation - Adult

The cellular production and expression of CGRP with respect to injury is complex and is influenced by the type of injury as well as changes in afferent input. When afferent input is interrupted, major

changes occur with respect to the input of signals to motoneurons. Transection of the spinal cord results in degeneration of supraspinal projections to motoneurons below the level of the lesion. The cortico-, tecto-, rubro-, and bulbo-spinal tracts are the main supraspinal fiber tracts projecting to the spinal cord (Piehl et al., 1991). Contacts with motoneurons are also made via interneurons which also receive supraspinal influence (Marlier et al., 1990). Typically, spinal cord deafferentation in the adult rat results in an overall decrease in CGRP-IR (Arvidsson et al.,1989; Piehl et al., 1991). After spinal cord transection, CGRP appears altered within 48 hours. Maximal reduction in CGRP-IR occurs within the first month (Marlier et al., 1990). From 3 to 5 months postoperative, the number of CGRP immunoreactive cells and the intensity of the immunocytochemical staining increases resulting in a partial recovery to normal levels (Arvidsson et al., 1989). Decreased CGRP expression after spinal cord transection may be due to the presence of a factor which inhibits or suppresses CGRP synthesis in motoneurons or it may be caused by an interruption of the descending input itself (Piehl et al., 1991). The direct removal of some crucial substance normally acting on the motoneuron or indirectly changing the activity pattern of the motoneuron may also result in decreased CGRP expression (Arvidsson et al., 1989). Interestingly, when a peripheral nerve transection is performed on a rat that has had a spinal cord transection, CGRP returns to normal levels in the dorsal horn of the spinal cord. This response may be a feedback response in which the inhibitory factor which suppressed the CGRP response is absent (Arvidsson et al., 1989) or the up-regulation of

CGRP may be controlled by different cellular mechanisms which override the effect of a spinal cord transection (Piehl et al., 1991).

The changes observed in CGRP following deafferentation can be compared to those observed in the dorsal root ganglion (DRG) after peripheral nerve injury. Generally, CGRP-IR is colocalized in sensory neurons containing Substance P (Dumoulin et al., 1991). Following sciatic nerve injury, CGRP immunocytochemical levels in DRG sensory neurons decrease significantly by 5 dpo. By the second postoperative week, the amount of CGRP is maximally decreased reaching 45% of the level of CGRP in the control DRG which persist through 45 days postoperative (Dumoulin et al., 1991). Substance P shows a similar decrease after axotomy (Dumoulin et al., 1991).

Interestingly, following dorsal rhizotomy an increase in CGRP and Substance P immunoreactivity is observed in the DRG. This increase is observed from 1-15 dpo (Inaishi et al., 1992). As was seen following double injury, after spinal cord transection, normal levels of CGRP-IR are observed when a dorsal rhizotomy is performed on a rat that has had a sciatic nerve transection 1 week previously (Inaishi et al., 1992). These differences would suggest that CGRP expression in sensory neurons may be regulated by the action of factors via central processes and that injury in different areas of the nervous system can either stimulate or inhibit the release of factors which effect CGRP expression.

CGRP Response to Denervation of Motoneurons - Adult

CGRP peptide and α and β CGRP mRNA levels are maintained at low levels in intact, adult motoneurons unless denervation occurs (Streit et al., 1989; Haas et al., 1990) or axonal transport is interrupted (Réthelyi et al., 1991). Nerve crush, transection, or resection of cranial or spinal motoneurons produces rapid increases in α and β CGRP mRNA (Noguchi et al., 1990; Dumoulin et al., 1991; Saika et al., 1991a) that precede marked elevations of peptide expression. CGRP-IR reaches maximal levels at 3 days post injury (Streit et al., 1989; Arvidsson et al., 1990; Haas et al., 1990; Borke et al., 1993). The paradigm of later changes in CGRP mRNA (Haas et al., 1990; Noguchi et al., 1990; Dumoulin et al., 1991; Saika et al., 1991a) and peptide, however, are dependent upon the type of injury (Borke et al., 1993). Increased expression of α CGRP mRNA and peptide is sustained after nerve crush until reinnervation occurs, whereas, transient reductions of β CGRP mRNA (Noguchi et al., 1990; Saika et al., 1991a) and peptide expression occur after transection of the facial (Dumoulin et al., 1991) and transection (Borke et al., 1993; Grothe, 1993) or resection of hypoglossal nerves (Borke et al., 1993). In the hypoglossal nucleus the reduction in the peptide content is followed by a second increase, which begins earlier after transection than after resection (Borke et al., 1993). The differences in α and β gene expression after injury suggest that different mechanisms may control the synthesis of α and β CGRP. It has been suggested that in

motoneurons α CGRP has a trophic action and β CGRP is involved in neural transmission (Saika et al., 1991a).

The reason for the increase in CGRP synthesis in response to axotomy has not yet been determined but increased levels of CGRP in regenerating motoneurons suggests a supportive role for the peptide in neuronal regeneration (Dumoulin et al., 1992). Changes in the level of CGRP in the motoneurons may be under the control of the NMJ (Arvidsson et al., 1990). Injury may interrupt a retrograde signal at the NMJ; and this may trigger an acute increase in the production of CGRP (Arvidsson et al., 1990). The CGRP released at the injury site may have an effect either on Schwann cells, macrophages, and/or fibroblasts, or it may assist the axon, helping it to find its path to the denervated muscle (Arvidsson et al., 1990). CGRP may also help to promote regeneration by increasing blood flow at the injury site as it is known to be a potent vasodilator (Brain et al., 1985). Finally, CGRP could be important in the survival of the injured motoneuron itself to assist in regeneration. The increase in CGRP may have an effect around the cell body and dendrites by acting on glial cells (Haas et al., 1990). This is supported by the fact that the peak observed in the biphasic pattern corresponds to the early and delayed astrocytic reaction observed in the facial nucleus (Dumoulin et al., 1991).

Enhanced CGRP expression during development and in response to motoneuron axotomy emphasizes the role of CGRP in modulating trophic

functions. To date, no study has investigated what changes in α CGRP mRNA and its peptide are produced by motoneuron denervation during postnatal development. Since CGRP expression is transiently up-regulated postnatally (Kubota et al., 1988; Hares & Foster, 1991), it would be of interest to determine if there is a difference in CGRP expression after nerve injury in rats of increasing postnatal ages. If there is a difference, then, when during development do the changes in CGRP expression mimic those elicited in adult rats by the same types of nerve injury? Finally, can an upregulation of CGRP after axonal injury to motoneurons during postnatal development be correlated with neuronal survival and reinnervation of target musculature as has been demonstrated in adult motoneurons?

Choline Acetyltransferase

Choline acetyltransferase (ChAT) is the key enzyme in the synthesis of the neurotransmitter, acetylcholine, and is a specific marker for cholinergic neurons (Houser et al., 1983; Phelps et al., 1984; Wainer et al., 1984). Determination of ChAT immunoreactivity (ChAT-IR) and enzyme activity has been widely used to localize cholinergic neurons in the CNS and to study their functions in different nerve injury paradigms. (Phelps et al., 1984). Levels of ChAT in the medulla and pons exceed all other CNS regions of the adult rat brain (Ibáñez et al., 1991). Specifically, the hypoglossal nucleus, comprised chiefly of large, cholinergic motoneurons,

contains the highest adult level of ChAT activity (Kobayashi et al., 1975). Biochemically, ChAT content in the adult hypoglossal nucleus is two times higher than in the dorsal motor nucleus and four times higher than levels in other nuclei such as the nucleus of the solitary tract, the spinal trigeminal nucleus, as well as the medullary reticular nuclei: pars ventralis and pars dorsalis (Kobayashi et al., 1975). For this reason, the hypoglossal nucleus provides an attractive model to study injury-induced changes in ChAT expression. In the hypoglossal nucleus, ChAT is synthesized in the nerve cell bodies and is transported unidirectionally to axon terminals in the tongue at a rate of 2-17 mm/day where it assists in the production of ACh (Tucek, 1990).

Ontogeny of ChAT in the CNS

ChAT activity is known to be developmentally regulated with the emergence of cholinergic neurons at different times in different brain regions (Phelps et al., 1984). Differences in the timing and intensity of ChAT expression throughout the CNS may be correlated to the maturation of cholinergic synaptic activity which is manifested by individual cells or groups of neurons (Ibáñez et al., 1991). ChAT expression during development appears in a caudal to rostral fashion. In the rat spinal cord, ChAT mRNA has been detected as early as E13 until birth (Ibáñez et al., 1991), increasing to adult levels by 3-4 postnatal weeks (Phelps et al., 1984). In the cortex and hippocampus, a sharp increase in ChAT activity

occurs within 10 days after birth reaching adult levels by the third postnatal week (Large et al., 1986).

Response of ChAT to Denervation - Adult

Reductions in ChAT after axotomy have been documented by biochemical (Wooten et al., 1978) and immunocytochemical (Lams et al., 1988; Armstrong et al., 1991; Borke et al., 1993; Chiu et al., 1994; Rende et al., 1995) studies. In adult hypoglossal motoneurons, there is a transient decrease in ChAT-IR that is influenced by the type of nerve injury. A modest, short term reduction in ChAT-IR follows nerve crush (Armstrong et al., 1991; Moix et al., 1991; Borke et al., 1993; Rende et al., 1995), whereas nerve transection (Armstrong et al., 1991; Borke et al., 1993; Rende et al., 1995) or nerve resection (Borke et al., 1993) results in an almost complete disappearance of ChAT staining for several weeks. Although the significance of the decrease is not clear, it is postulated to be related to a reordering of the metabolic priorities in the injured neuron so that the synthesis of neurotransmitter-related enzymes is decreased and the expression of structural proteins for axonal regrowth is augmented (Matthews & Raisman, 1972; Svensson & Adlskogius, 1993).

In all of the injury paradigms, the effects on ChAT-IR are reversible and reinnervation is not necessarily required for the restoration of normal, noninjured ChAT levels (Borke et al., 1993; Chen et al., 1995). However, the return of ChAT to normal levels was gradual and associated with the

type of injury. The recovery of ChAT to normal levels was related to the timing of reinnervation and occurred by 20 dpo after nerve crush (Borke et al., 1993). For nerve transected and resected rats, recovery of ChAT to normal levels at 50 dpo did not coincide with reinnervation of lingual musculature (Borke et al., 1993). This target-independent behavior in the return of ChAT after nerve injury, is reminiscent of the initial induction of ChAT expression in development; spinal motoneurons in the rat embryo express immunoreactivity before target contact is made (Phelps et al., 1994, 1991). In these motoneurons, levels of ChAT continue to increase during the early postnatal period, when transmitter release from the nerve terminals is augmented (Phelps et al., 1984), suggesting that maintenance of transmitter phenotype is influenced postnatally by nerve-target interaction.

Application of vincristine, an inhibitor that blocks axonal transport also produces decreased ChAT expression in hypoglossal motoneurons (Moix et al., 1991; Greeson et al.,1992). Additionally, it has been shown that decreased ChAT expression can not be used as an index for predicting injury induced cell death (Lams et al., 1988). The fact that the disappearance of ChAT-IR does not successfully predict cell death or successful reinnervation (Lams et al., 1988; Borke et al., 1993) supports the premise that enzymes involved with intracellular transmission undergo substantial variation and that their expression can be regulated independent of factors that determine neuronal survival or degeneration following injury (Lams et al., 1988).

ChAT Study - Current Investigation

Postnatal development is characterized by alterations in the expression of enzymes and peptides as the priorities of the motoneuron mature switch from growth to neural transmission. During development, levels of ChAT-IR in the spinal cord increase and do not reach adult levels until 21-28 dpn (Phelps et al., 1984). This increase coincides with a period of substantial development of motor function. A study detailing the postnatal changes in ChAT and the response of this enzyme to different types of nerve injury during postnatal development would be of interest in correlating the axotomy-induced response of co-localized molecules ACh and CGRP.

Low Affinity Nerve Growth Factor Receptor

Neurotrophic factors are necessary for the normal development and maintenance of the central and peripheral nervous systems. Neurotrophins belong to the classification of neurotrophic factors which are retrogradely transported from the CNS or from peripheral targets to assist in the survival and maintenance of neurons expressing their specific receptors. The neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) (Barde, 1989, Glass & Yancopoulos, 1993).

Two types of receptors bind the neurotrophin family; a high affinity form and a low affinity form. The effects of neurotrophins are mediated through interaction with their specific high affinity receptors (DiStephano et al., 1992). Members of the tyrosine kinase (Trk) family of receptors: Trk A, Trk B, and Trk C encode the essential components of the high affinity receptors for NGF, BDNF, and NT-3, and NT-4/5 and mediate their binding, uptake, and retrograde transport *in vivo* (DiStephano et al., 1992). NGF mediates its effects through Trk A; BDNF and NT-4/5 via Trk B; and NT-3 interacts via mainly Trk C but can also bind to Trk A and Trk B (DiStephano et al., 1992, Wong et al., 1993). High affinity receptors can readily discriminate between the neurotrophins and only bind to its specific neurotrophin (Rodriguez-Tébar, 1992). All members of the neurotrophin family are capable of binding to the low affinity neurotrophic receptor, known also as low affinity nerve growth factor receptor (LNGFR) with similar affinity (Rodriguez-Tébar, 1992).

Ontogeny of LNGFR in Motoneurons

Developing brainstem and spinal motoneurons are known to express LNGFR but not the high affinity form of the receptor (Yan & Johnson, 1988). Typically, LNGFR is expressed in brainstem and spinal motoneurons between E13-18 (Yan & Johnson, 1988, Ernfors et al., 1989), peaks at birth (Yan & Johnson, 1988), and disappears during the final stages of maturation over the second postnatal week (Yan & Johnson, 1988; Ernfors et al., 1989; Chiu et al., 1993). It is thought that the

presence of LNGFR is associated with target innervation and synapse formation (Yan & Johnson, 1988; Ernfors et al., 1989). Mature motoneurons retain the ability to express LNGFR and reexpression is seen following injury (Ernfors et al., 1989; Wood et al., 1990; Armstrong et al., 1991; Koliatsos et al., 1991; Moix et al., 1991; Saika et al., 1991b; Greeson et al.,1992; Hayes et al., 1992; Rende et al.,1992; Chiu et al., 1993, Rende et al., 1993; Rende et al., 1995).

Response of LNGFR to Denervation of Motoneurons - Adult

Nerve crush in adult rats produces an intense LNGFR reaction in spinal and brainstem motoneurons (Wood et al., 1990; Saika et al., 1991b; Hayes et al., 1992; Rende et al.,1995); whereas nerve transection produces an LNGFR response that is longer in duration compared to crush injury (Wood et al., 1990; Armstrong et al., 1991; Saika et al., 1991b; Hayes et al., 1992; Rende et al.,1995). This effect is opposite to that seen in sensory neurons where LNGFR is expressed under normal conditions, down regulated after nerve injury, and recovered after nerve regeneration (Verge et al., 1989). Although the function of LNGFR in developing and regenerating motoneurons is not known (Yan et al., 1988), its expression appears to be associated with periods of axonal growth and maturation.

The signal for the induction of LNGFR following peripheral nerve injury is dependent on a signal from the injury site. This is evident based on studies documenting the failure of LNGFR to be reexpressed when

axonal transport is blocked chemically (Moix et al., 1991; Greeson et al.,1992; Hayes et al., 1992), or substances are applied that are known to cause toxic nerve damage (Rende et al., 1993), or the proximal end of a transected nerve is ligated after injury (Wood et al., 1990).

The essential role of neurotrophic factors in motoneurons during development and after nerve injury is support and maintenance. Since the classic neurotrophic effects of NGF are not observed when it is bound to the low affinity receptor and since the high affinity type is not present in motoneurons after injury this may explain why the classic NGF response is not observed (Wood et al., 1990) and suggests that perhaps some other neurotrophic factor may bind to LNGFR such as BDNF, NT-3, or NT-4/5 (Barde, 1989; Rodriguez-Tébar et al., 1990, 1992; Glass & Yancopoulos, 1993; Yan et al., 1993).

While motoneurons fail to express the Trk A receptors they are capable of expressing Trk B and Trk C mRNAs during development (Yan et al., 1993) and into adulthood (Koliatsos et al.,1993;Yan et al., 1993). Furthermore, target muscle cells normally contain BDNF and NT-3 (Henderson et al., 1993) and nerve injury up-regulates the expression of BDNF and NT-4/5 in nonneuronal cells surrounding the distal ends of injured sciatic nerve (Meyer et al., 1992; Funakoshi et al., 1993). Based on recent studies demonstrating that LNGFR can function as an auxillary molecule for Trk B it has been suggested that the increased expression of

LNGFR following peripheral nerve injury may enhance the sensitivity of motoneurons to BDNF and NT-4/5 (Friedman et al., 1995a).

These factors may also have putative neurotrophic effects on motoneurons since labeled BDNF and NT-3 injected into the muscle (Yan et al., 1988; Yan et al. 1993) and BDNF and NT-3 injected into the crushed sciatic nerve (DiStephano et al., 1992) can be retrogradely transported. Also, local application of BDNF, NT-3, NT-4/5 have been shown to promote motoneuron survival (Yan et al.,1992; Koliatsos et al., 1993; Wong et al., 1993; Yan et al.,1993).

LNGFR - Current Study

The reason for studying LNGFR is that it is another molecule whose expression changes during development and in response to denervation. It is the only one of the three molecules I will study whose developmental production ceases postnatally and is re-synthesized only if adult motoneurons are denervated. Therefore, it would be of interest to follow the timing of the transient expression of LNGFR postnatally and to see if this surface molecule is upregulated in motoneurons after different types of nerve injury during postnatal development.

CHAPTER 2.

Calcitonin Gene-Related Peptide and α CGRP mRNA Expression in Cranial Motoneurons After Hypoglossal Nerve Injury During Postnatal Development

Materials and Methods

Animal Population

Two hundred and seventy-six Sprague-Dawley rats of both sexes were used for this investigation. Three different methods were utilized for the experimental and control series: CGRP immunocytochemistry, α CGRP ribonuclease protection assay (RPA) and a regeneration assay using retrograde transport of horseradish peroxidase (HRP). The experimental series for CGRP immunocytochemistry consisted of 144 rats. Hypoglossal nerve crush or nerve transection was performed in four animals at each of three postnatal ages (10, 14, and 21 dpn at six different survival times (1,3,7,14,20 and 40 dpo). A developmental series for CGRP immunocytochemistry consisted of four normal rats at seven different postnatal ages (3, 7,10, 14, 17, 21, and 30 dpn), as well as adult (200-250 gm) for a total of 32 rats. For the RPA experimental series for α CGRP mRNA, four rats per injury of two age groups (10 and 21 dpn) were

euthanized at three survival periods (<1, 6, and 18 dpo) for a total of 48 rats. The developmental RPA series for α CGRP mRNA consisted of 12 normal, noninjured rats: four rats at three different postnatal ages (10, 14, and 21 dpn). The HRP regeneration assay was carried out in 40 rats. For the three postnatal age groups (10, 14, and 21 dpn), two rats were euthanized at 14, 20 and 40 days after nerve crush or transection. Two additional rats were euthanized at 3 and 7 dpo after nerve crush in 21 dpn rats.

Surgical Procedures

Early postnatal rats are insensitive to small dosages of most injected anesthetics (Borke, 1982). Therefore, 10 and 14 dpn rats were anesthetized with ether. For rats 21 dpn or older, either 7% chloral hydrate (0.5 ml per 100 g) or a ketamine/rompun combination (ketamine [50 mg/kg]; rompun [10 mg/kg]) was used. One of two types of nerve injury was performed on the right hypoglossal nerve proximal to its bifurcation into medial and lateral branches. The nerve injuries consisted of either a nerve crush for three minutes using a self-locking needle holder with smooth jaws or a nerve transection with apposition of the cut ends.

Following surgery the pups were returned to their dams. Pup weights were recorded daily. Supplemental gavage feeding and soft foods were provided to ensure appropriate weight gain in the young animals.

Twenty-one day and older rats were kept to a standard *ad libitum* diet of pellets and water.

Immunocytochemistry

The rats were reanesthetized at the appropriate postoperative times and perfused transcardially with a fixative of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were removed and postfixed for two hours (h). The medullas were infiltrated overnight in 30% sucrose in 0.1 M phosphate buffer at 4° C, freeze-thawed in isopentane and cut at 40 μm transverse sections on a freezing microtome. Sections of the medulla through the rostro-causal extent of the hypoglossal nucleus were collected in Tris-buffered saline (TBS; pH 7.6). Free-floating sections were used for CGRP immunocytochemistry. Sections were pretreated with 10% normal goat serum (NGS; Cappel) in TBS for 1 h and incubated for 21 h at room temperature in a polyclonal rabbit CGRP antiserum (1:2500; Cambridge Research Biochemicals) in TBS with 20% NGS. Sections were washed in TBS and incubated for 2 h at room temperature in biotinylated goat anti-rabbit IgG (1:200; Vector) in TBS containing 1.5% NGS. After a TBS wash, the sections were incubated at room temperature in ABC ELITE Standard complex (1:100; Vector) for 2 h. Immunoreactivity was visualized with 3-3'diaminobenzidine (DAB; 0.06%) and H_2O_2 (0.005%) in 0.1 M phosphate buffer. Sections were mounted on glass slides, air dried, and dehydrated, cleared, and

coverslipped with Permount. Controls for the immunocytochemical procedures were processed by omitting the specific primary antiserum or by using antisera preabsorbed with an excess of the immunogen. No positive staining was detected after either treatment.

Immunocytochemical Analysis

The levels of CGRP-IR were assessed quantitatively with the aid of a computerized densitometry analysis system using NIH Image 1.54 linked to an Olympus microscope. For each case, four sections of the hypoglossal nucleus were randomly selected through the center of the hypoglossal nucleus for image analysis. Each section was digitized at a final magnification of 40 x using a MTI CCD 72 camera and a Scion LGF image grabber card in a Macintosh Quadra 800 (Apple Computer, USA). The gray scale used ranged from 0 for white to 255 for black. The final background level was determined from an unlabeled region of the adjacent reticular formation. Using a box measuring 50 x 50 pixels, mean pixel intensities were determined and averaged from three different areas of the nucleus (neurons plus neuropil) on the right and the left sides. The mean values from four cases/developmental group were converted to right/left (R/L) ratios and mean ratio values were used to determine the percent change in immunocytochemical staining intensity for the antigen. These values from each injured treatment group were then compared to the data for the non- injured group for each age group by a one way ANOVA. The

Student Newman Keuls test was applied *post hoc* to inspect differences between means when F values from ANOVA produced $p< 0.05$ (Snedecor & Cochran, 1980). All computations were performed with the Graph Pad Instat statistical package.

Ribonuclease Protection Assay

Probe Synthesis

An α CGRP RNA probe template was generated by PCR amplification. A 439 base pair fragment was obtained using specific α CGRP oligonucleotide primers (sense [5'-TCAACCTTAGAAAGCAC GCC-] and antisense [-TAGGGAGAAGGGTTTCAGTACC-3'], Amara et al., 1984) that were synthesized on an ABI 392 synthesizer (Applied Biosystems, Inc.). This product was reamplified using the same primer set with the antisense primer containing the T_7 RNA polymerase sequence (Amersham). The control probe for glyceraldehyde 3-phosphate-dehydrogenase (GAPDH; Fort et al., 1985) was purchased from Ambion. Both the α CGRP and GAPDH RNA probes were synthesized using [α-^{32}P]-rUTP (800 Ci/mmol, Amersham) according to the manufacturer's instructions. The labeled RNA probe was separated on a 6% urea/acrylamide gel, isolated, purified, and used in subsequent hybridizations (Potts et al., 1990).

RNA Isolation and Hybridization

Intact and experimental rats were euthanized by decapitation and hypoglossal nuclei were isolated for the preparation of RNA. RNA was purified (Chomczynski & Sacchi, 1987) from four whole hypoglossal nuclei. Total RNA (equivalent to two whole nuclei) was hybridized with either α CGRP or GAPDH probes as described by manufacturer's instructions using the Hyb Speed RPA kit (Ambion). The RNA/probe mixture was precipitated and subjected to electrophoresis on a 6% urea/acrylamide gel. The gel was dried under a vacuum gel dryer and exposed to Kodak X-OMAT AR film overnight at -20^0 C using intensifying screens.

RPA Analysis

Autoradiograms were scanned on a laser scanner (UMAX UC1260, Data Systems) and the images were digitized using Metroscan 2.5 (HSD microcomputer US, Inc. [1994]). The gel analysis macro in NIH Image 1.54 was used to determine the size (volume) of the bands on the electrophoresis gels. The volume was estimated by calculating the integrated density. Integrated density was computed using the following formula: Integrated Density = N * (Mean- Background). N = the number of pixels in a defined area, Mean = the mean pixel value and Background = the most common pixel value measured in the defined area. To assure

that the differences in α CGRP mRNA values were not due to variations in the amount of RNA loaded per lane, the integrated densities were normalized by determining the ratio of the α CGRP bands versus GAPDH bands. GAPDH mRNA has been shown to remain relatively constant in rat peripheral nerve during postnatal development and in response to nerve crush or transection in the adult (Scherer et al., 1994). Because of the relative constancy of GAPDH mRNA during the postnatal periods examined in the current study, this transcript was chosen as our standard. The effects of the injury were assessed by calculating the ratio of the integrated densities for α CGRP/GAPDH in RNA samples of nuclei from the injured rats divided by the integrated densities for α CGRP/GAPDH in the nuclei of the noninjured rats (Haas et al., 1993).

Reinnervation and Neuronal Survival

To assay reinnervation of the tongue musculature and neuronal survival, the same two injuries were performed in two rats at each of the three postnatal ages (10, 14, and 21 dpn). These rats survived for 14, 20, or 40 days. In addition, for the reinnervation assay, two rats were examined at 3 and 7 days after nerve crush to 21 dpn rats. At 36 and 24 h before euthanizing, the animals were reanesthetized and a 10% solution of HRP (Boehringer Mannheim) in 0.1 M Tris buffer (pH 7.6) was injected (approximately 10 μl per injection) with a Hamilton syringe into three

different areas of the tongue musculature on the right and left sides. The rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) followed by a post wash of 10% sucrose in 0.1 M phosphate buffer. The medulla was isolated and infiltrated overnight in 30% sucrose at 4^0 C. Transverse sections were cut at 40 μm using a freezing microtome and collected in 0.1 M Tris buffer. Free-floating sections throughout the length of the medulla were processed by the DAB-glucose oxidase method (Itoh et al., 1979) and counterstained with Neutral Red.

Reinnervation Analysis

The number of HRP-labeled and unlabeled hypoglossal neurons was counted in injured and intact nuclei in every sixth section through the rostro-caudal extent of the hypoglossal nucleus. HRP labeling was defined as the percentage of labeled neurons in the total neuronal population of each hypoglossal nucleus. The percentages of labeled neurons in the total neuronal population of the right and left hypoglossal nuclei were compared to assess reinnervation.

Neuronal Survival Analysis

A ratio of the number of HRP-labeled and unlabeled hypoglossal neurons in the injured nuclei versus the number of neurons in the

noninjured nuclei was converted to a percent and used to estimate frequency of neuronal survival after the two types of nerve injury. Only those neurons which contained a nucleus were counted. For each nerve injury case, the total number of neurons in each hypoglossal nucleus was calculated from every sixth section of the hypoglossal nucleus from the rostral to caudal extent according to the following formulas (Koningsmark, 1970):

Total Neuronal Number = sum of all neurons counted x (total section number/total number of sections counted)

Total Section Number = [(total number of sections counted - 1) (section interval)] + (total number of sections counted).

The mean and the standard deviation were calculated for two rats/group. These means were compared for each survival period by a one way ANOVA. The Student Newman Keuls test was applied *posthoc* to inspect differences between means when F values from ANOVA produced p of less than 0.01 (Snedecor and Cochran, 1980). All computations were performed with the Graph Pad Instat statistical package.

Results

CGRP Immunoreactivity

Noninjured Hypoglossal Nuclei

Hypoglossal motoneurons were immunoreactive for CGRP at all postnatal ages of this study. Slight to moderate CGRP immunoreactivity was seen in the majority of dorsally located neuronal cell bodies in the hypoglossal nuclei of 3 and 7 dpn rats whereas neurons in the ventral portions of the nuclei demonstrated intense immunoreactivity (Figs. 1 A,B). The mean pixel intensity of CGRP-IR, nearly doubled from 7 to 10 dpn (Fig. 2 [$p<.001$]). This level of intensity of CGRP expression was maintained from 10 to 30 dpn (Fig. 2). During these postnatal periods, CGRP-IR was visible in hypoglossal perikarya and their processes (Figs. 1C-F). Decreased levels of CGRP immunoreactivity were detected in adult hypoglossal motoneurons compared to samples from 10 or 21 dpn rats (Figs. 1 C, D, E, F [$p<0.001$]). CGRP-IR in adult hypoglossal nuclei was similar in intensities to those estimated in hypoglossal nuclei of 3 and 7 day old rats (Fig. 2).

Figure 1. CGRP-IR in the hypoglossal nuclei of normal, noninjured rats: A: 3 dpn; B: 7 dpn; C: 10 dpn; D: 14 dpn; E: 21 dpn; F: adult.

Developmental Progression of CGRP

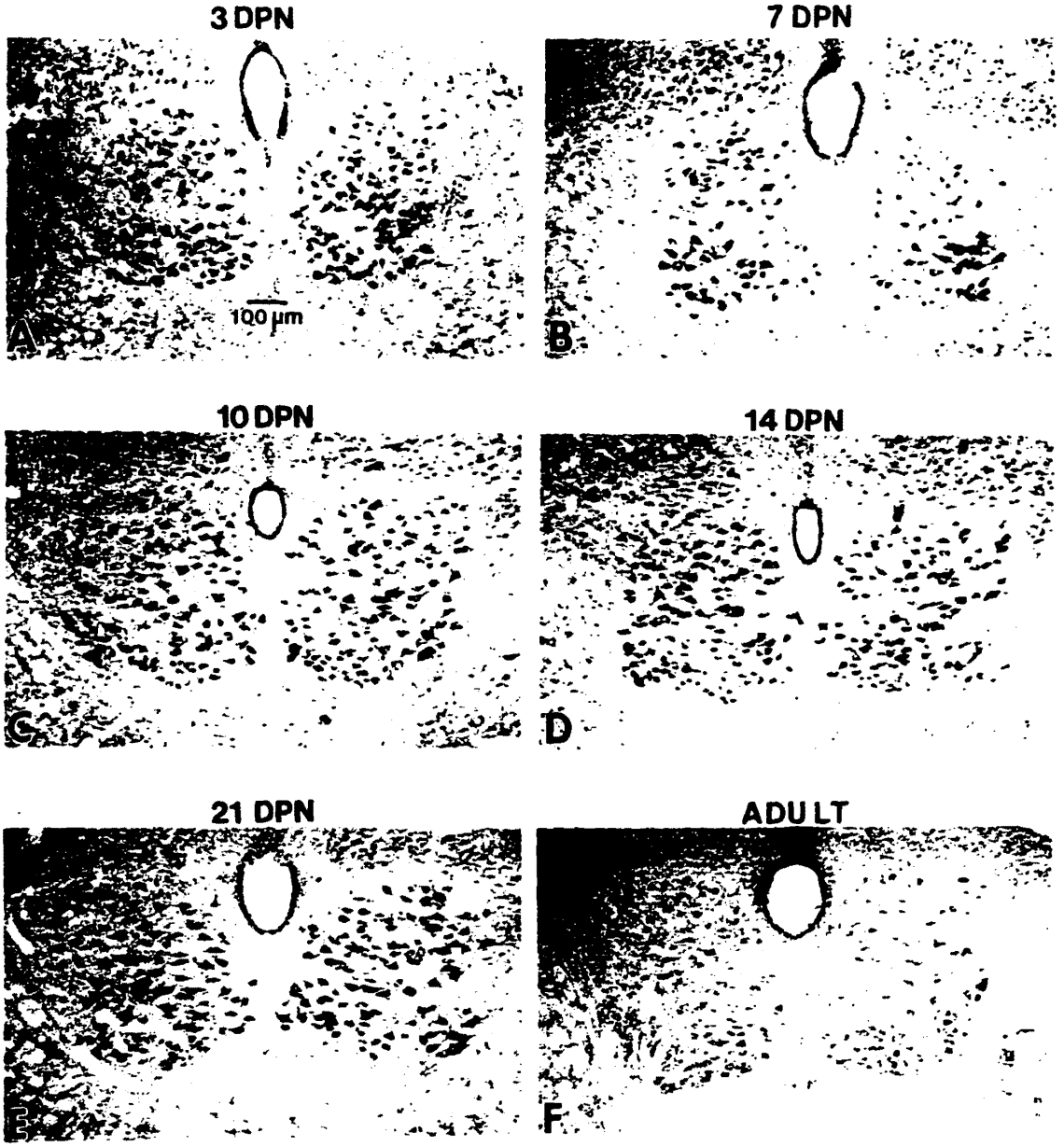
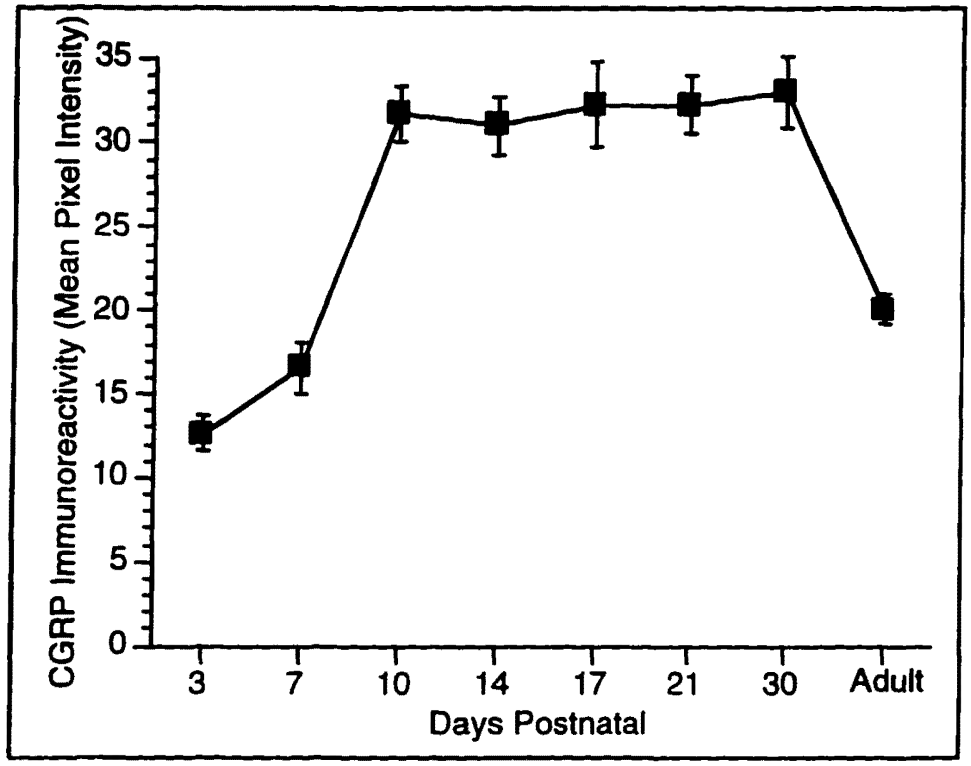


Figure 2. Change in mean pixel intensity of CGRP-IR in hypoglossal nuclei during postnatal development. Means (± SEM) are reported.


CGRP Immunoreactivity (Mean Pixel Intensity)
35
30
25
20
15
10
5
0
3
7
10
14
17
21
30
Adult
Days Postnatal

Injured Hypoglossal Nuclei

Nerve Crush

The CGRP response following nerve crush was dependent upon the postoperative age of the animal at the time of injury. Nerve crush in 10 dpn rats produced an ipsilateral reduction in CGRP-IR in hypoglossal motoneurons that was significant at 3 dpo (Fig. 3A [$p<0.05$]). This decrease in CGRP was noticed in neuronal cell bodies (Fig. 4A). Although a reduction in CGRP-IR appeared to persist until 7 dpo in 10 dpn rats, the values were not significantly different from the normal levels of intact hypoglossal nuclei (Fig. 3A). These normal levels of CGRP-IR continued in injured hypoglossal motoneurons from 14-40 dpo after nerve crush in 10 dpn rats (Figs. 3A & 4E,G).

There was no significant change in CGRP-IR in the hypoglossal nucleus ipsilateral to the nerve crush at any survival time for the 14 dpn cases (Fig. 3A). However, an apparent, transient increase in CGRP-IR was detected at 1-3 dpo in the neurons (compare Fig.5A to Figs. 5C,E,G).

An increase in CGRP-IR on the operated side was noticeable in 21 dpn rats as early as 1 dpo ($p<0.001$) and reached maximal levels at 3 dpo ($p<0.001$) (Fig. 3A) after nerve crush injury. CGRP-IR was abundant in neuronal cell bodies and their processes (Fig. 6A). The elevation in CGRP-IR persisted at 7 ($p<0.01$) and 14 dpo ($p<0.001$) (Fig. 3A)

Figure 3. Percent change in staining intensity of CGRP-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (A) or transection (B). Means (± SEM) are reported. Error bars are sometimes smaller than symbols and therefore are not always visible.

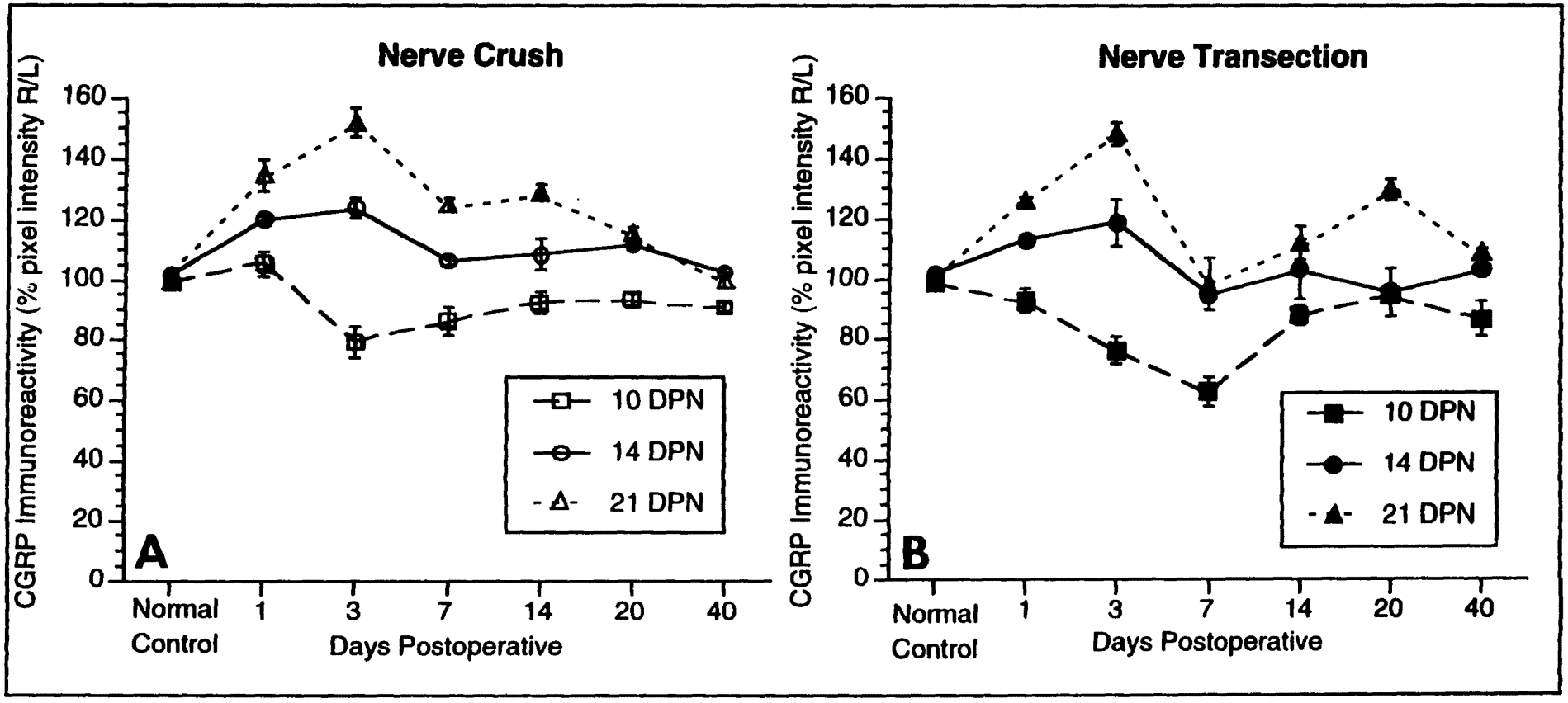

Nerve Crush
CGRP Immunoreactivity (% pixel intensity R/L)
160
140
120
100
80
60
40
20
0
10 DPN
14 DPN
21 DPN
A
Normal Control
1
3
7
14
20
40
Days Postoperative
Nerve Transection
CGRP Immunoreactivity (% pixel intensity R/L)
160
140
120
100
80
60
40
20
0
10 DPN
14 DPN
21 DPN
B
Normal Control
1
3
7
14
20
40
Days Postoperative

Figure 4. CGRP-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 10 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.

10DPN-CGRP

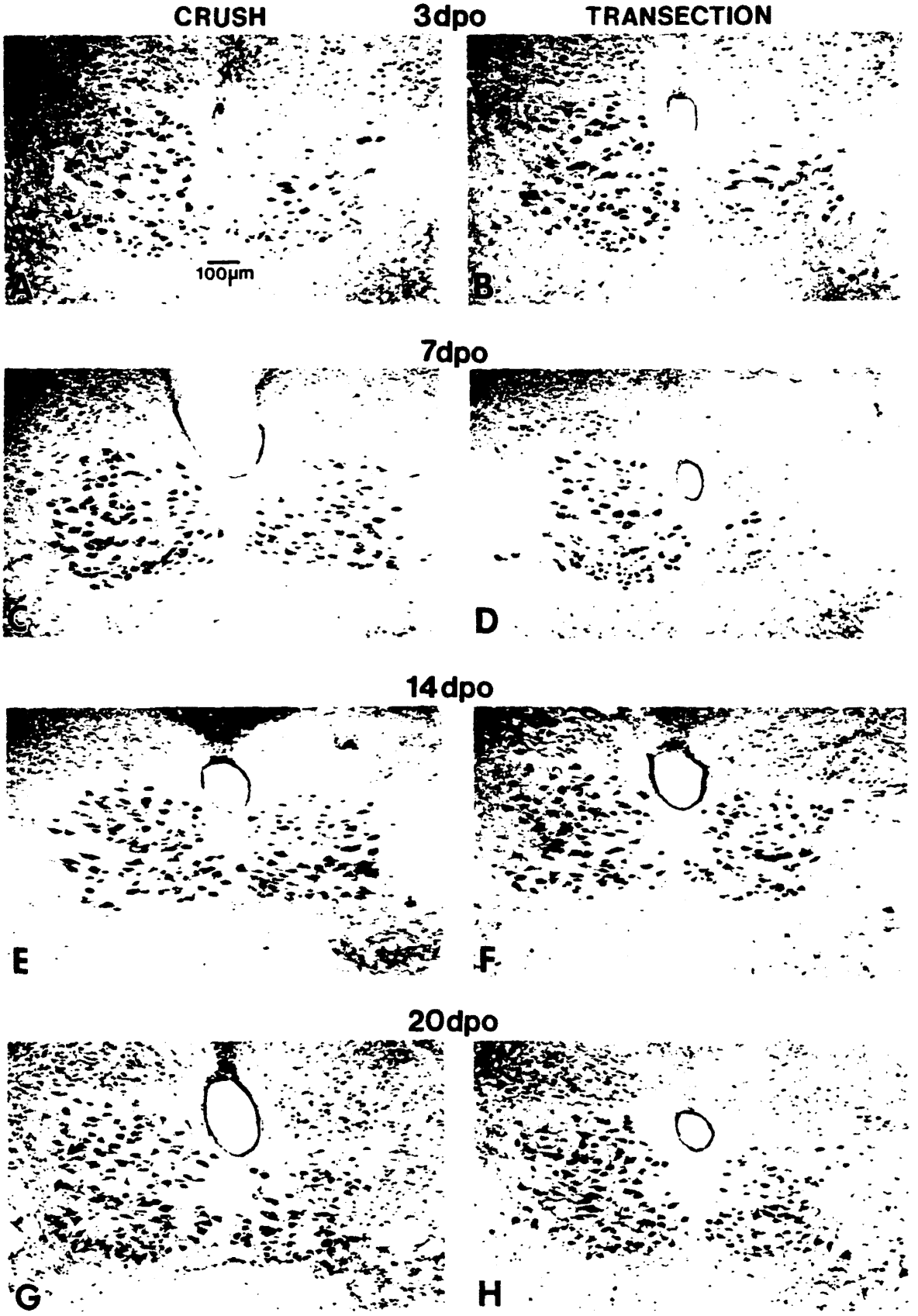


Figure 5. CGRP-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 14 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.

14 DPN-CGRP

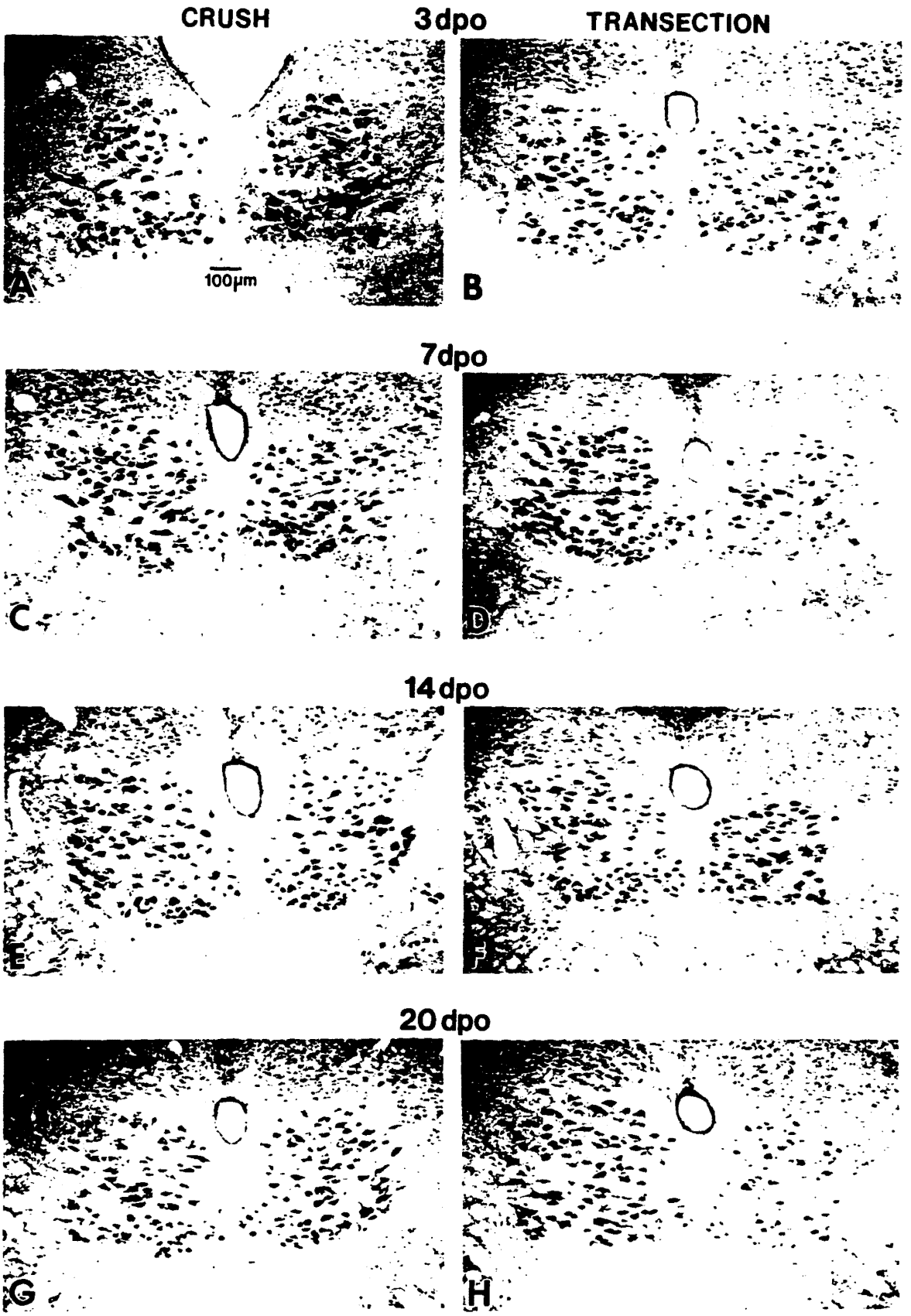
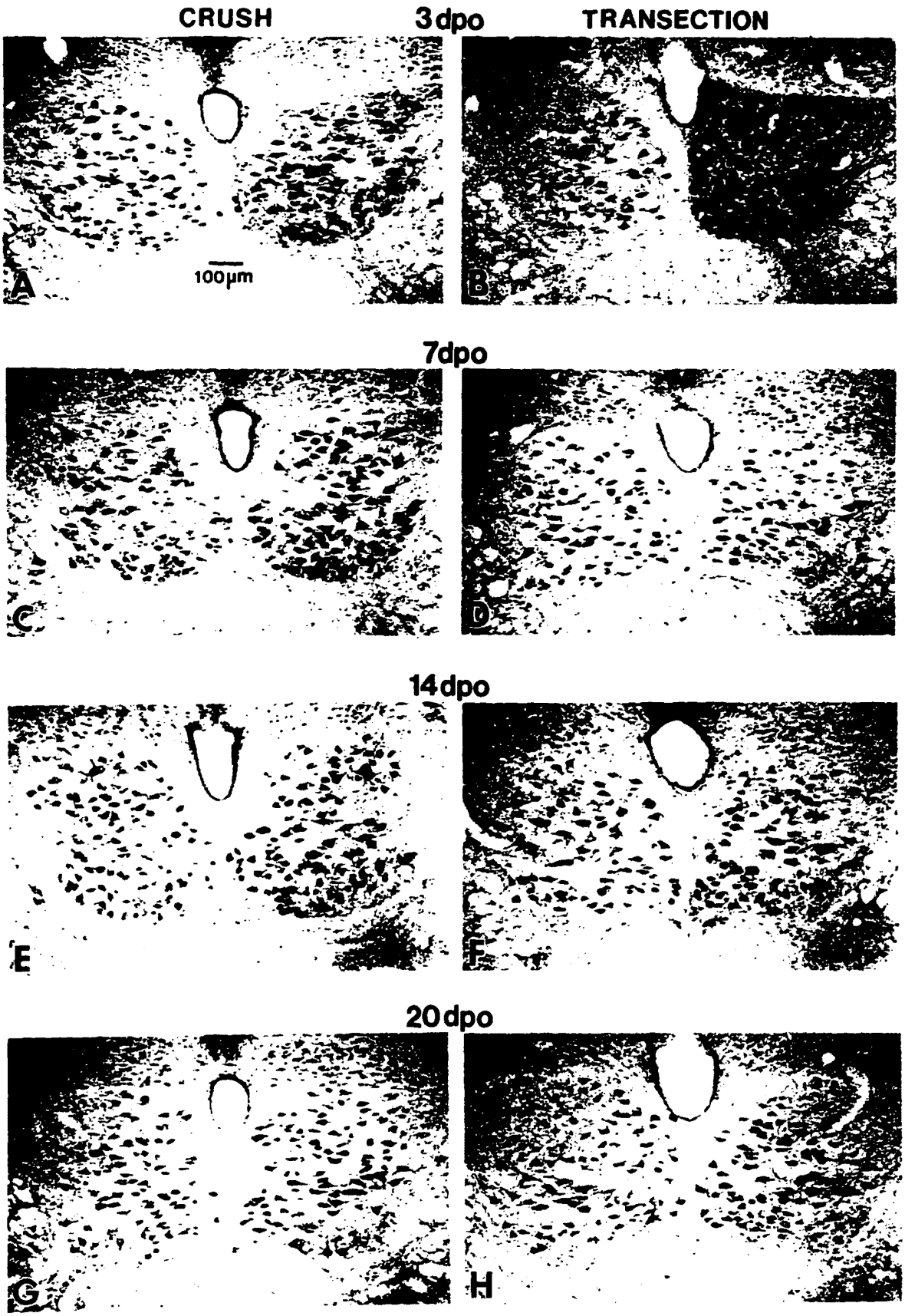


Figure 6. CGRP-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 21 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.

21 DPN-CGRP



in hypoglossal motoneurons (Figs. 6C,E) and returned to normal levels by 20 dpo (Figs. 3A & 6G) after nerve crush in 21 dpn rats.

Nerve Transection

The CGRP response to nerve transection was also dependent upon the postnatal age of the animal at the time of injury. Following transection in the 10 dpn rats, there was a decrease in CGRP-IR in the hypoglossal motoneurons (Figs. 4B,D). This decrease in CGRP-IR was significant at 3 dpo ($p<0.01$) and maximal at 7 dpo ($p<0.001$) (Fig. 3B). The mean pixel intensity of CGRP-IR at 7 dpo following nerve transection was, in fact, significantly less than those values obtained from tissue where rats sustained a nerve crush ($p<0.001$). A modest decrease in CGRP-IR persisted throughout the later postoperative periods [14, 20, and 40 dpo] (Figs. 4F,H) in the nerve-transected hypoglossal nucleus of 10 dpn rats, but these reductions were not significantly different from normal levels of CGRP-IR in intact hypoglossal nuclei (Fig. 3B).

Likewise, the slight reduction in CGRP staining noticed at 3, 7, and 20 dpo after nerve transection in the 14 dpn rats (Figs. 5B,D,H) was not consistent for all specimens (Fig. 3B). Neither was the apparent increase in CGRP-IR in the neurons of the ipsilateral hypoglossal nucleus at 14 dpo (Fig. 5F). At all postoperative periods examined, heterogeneity in

CGRP-IR occurred between rats after transection at 14 dpn compared to the same injury performed in rats 10 or 21 dpn.

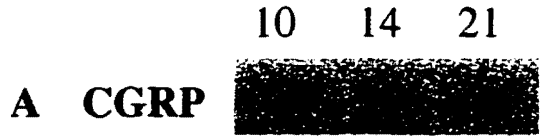
Following hypoglossal nerve transection in the 21 dpn rats, a biphasic response of CGRP-IR occurred. An increase was noticeable by 1 dpo ($p<0.01$) and maximal at 3 dpo ($p<0.001$) (Fig. 3B). CGRP reaction product filled the motoneurons, their processes, and the surrounding neuropil (Fig. 6B). A return to normal levels occurred by 7 dpo (Figs. 3B & 6D). A second elevation seemed apparent at 14 dpo (Figs. 3B & 6F), and was significant at 20 dpo ($p<0.01$) (Fig. 3B) when CGRP staining was abundant in the hypoglossal neurons (Fig. 6H).

Ribonuclease Protection Assay

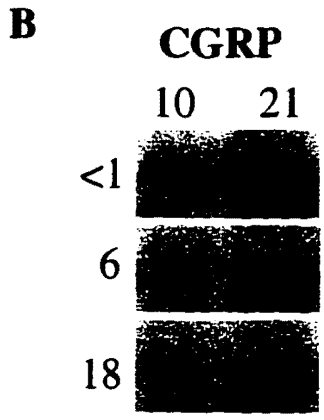
Noninjured Hypoglossal Nuclei

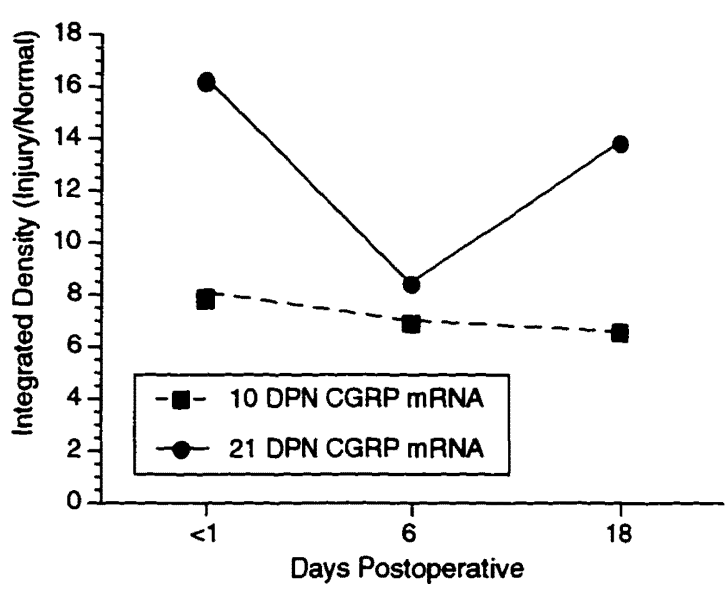
Hypoglossal nuclei expressed α CGRP mRNA at all postnatal ages examined in the current work. Comparable levels of α CGRP mRNA were expressed in the hypoglossal nuclei of normal noninjured rats at 10, 14, and 21 dpn (Figs. 7A,B). This postnatal interval corresponded to the time when peptide expression in the nucleus was maximal (Fig. 2).

Figure 7. Changes in α CGRP mRNA in hypoglossal nuclei during (A) postnatal development in 10, 14, and 21 day old normal rats and (B) after unilateral hypoglossal nerve transection and (C) nerve crush at <1, 6, and 18 days postoperative in 10 and 21 dpn rats. Values in Fig. 7D and 7E are expressed as a ratio of the integrated density in injured hypoglossal nuclei over the integrated density in noninjured hypoglossal nuclei after normalization for RNA amount loaded (see experimental procedures) for (D) nerve transection and (E) nerve crush. Fig. 7F summarizes measures of GAPDH mRNA in samples of tissue corresponding to normal noninjured rats at (1) 10 dpn and (2) 21 dpn, and after unilateral hypoglossal nerve transection at (3) 10 dpn, <1 dpo, (4) 21 dpn, <1dpo, (5) 10 dpn, 6 dpo, (6) 21 dpn, 6 dpo, (7) 10 dpn, 18 dpo, and (8) 21 dpn, 18 dpo. Similar GAPDH mRNA bands were observed following nerve crush (not pictured).

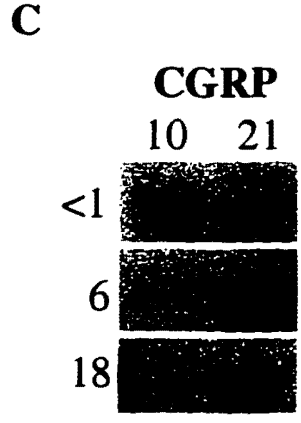


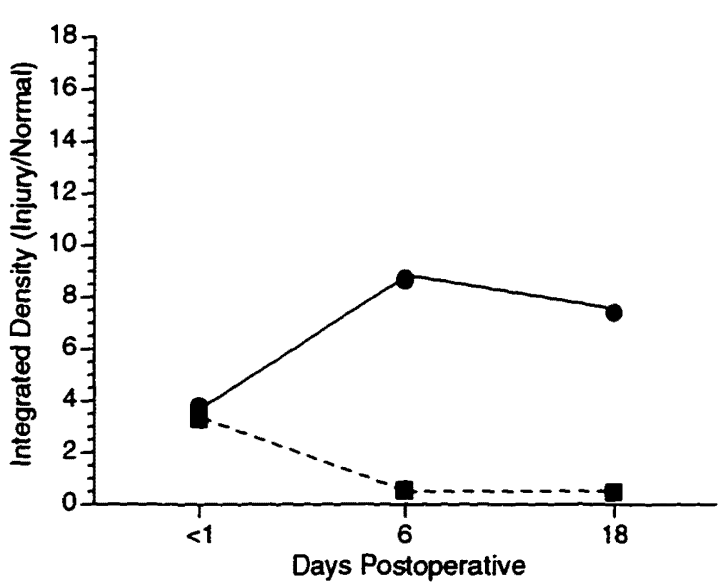
Nerve Transection

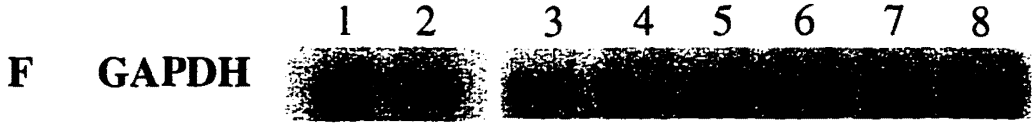




Nerve Crush







Injured Hypoglossal Nuclei

For the 10 and 21 day old rats, both types of injury induced an initial, rapid upregulation of α CGRP mRNA in injured hypoglossal nuclei compared to noninjured nuclei (Fig. 7A). The greatest elevation in the expression of α CGRP mRNA occurred rapidly (< 1 day, 15 h) in the 21 dpn rats after nerve transection (Figs. 7B,C). In these rats, by 6 dpo, gene expression had decreased to one-half the maximal levels (Figs. 7B,C) and a second elevation (Fig. 7B,C) occurred at 18 dpo. The biphasic increases in α CGRP mRNA after nerve transection in 21 dpn rats preceded the peptide increases detected at 1, 3 and 20 dpo (Fig. 3B). After nerve crush in the 21 dpn rats, a gradual up-regulation in α CGRP mRNA reached a maximal level at 6 dpo (Figs. 7D,E) that remained elevated at 18 dpo (Figs. 7D,E). This profile also fits well with the temporal progression of changes in peptide levels in the 21 dpn rats after nerve crush (Fig. 3A).

In 10 dpn rats, maximal elevations in α CGRP mRNA occurred in less than a day after both types of injuries (compare Figs. 7B,C & Figs. 7D,E). These elevations were less than those that were produced by the same injuries in the 21 dpn rats. For transection, the early elevation was one-half that obtained in the 21 dpn rats, but after nerve crush there was no difference in the levels of α CGRP mRNA expression compared to tissue obtained 21 day old rats (compare Figs. 7D & E). Only a slight decrease in gene expression was observed at later postoperative intervals

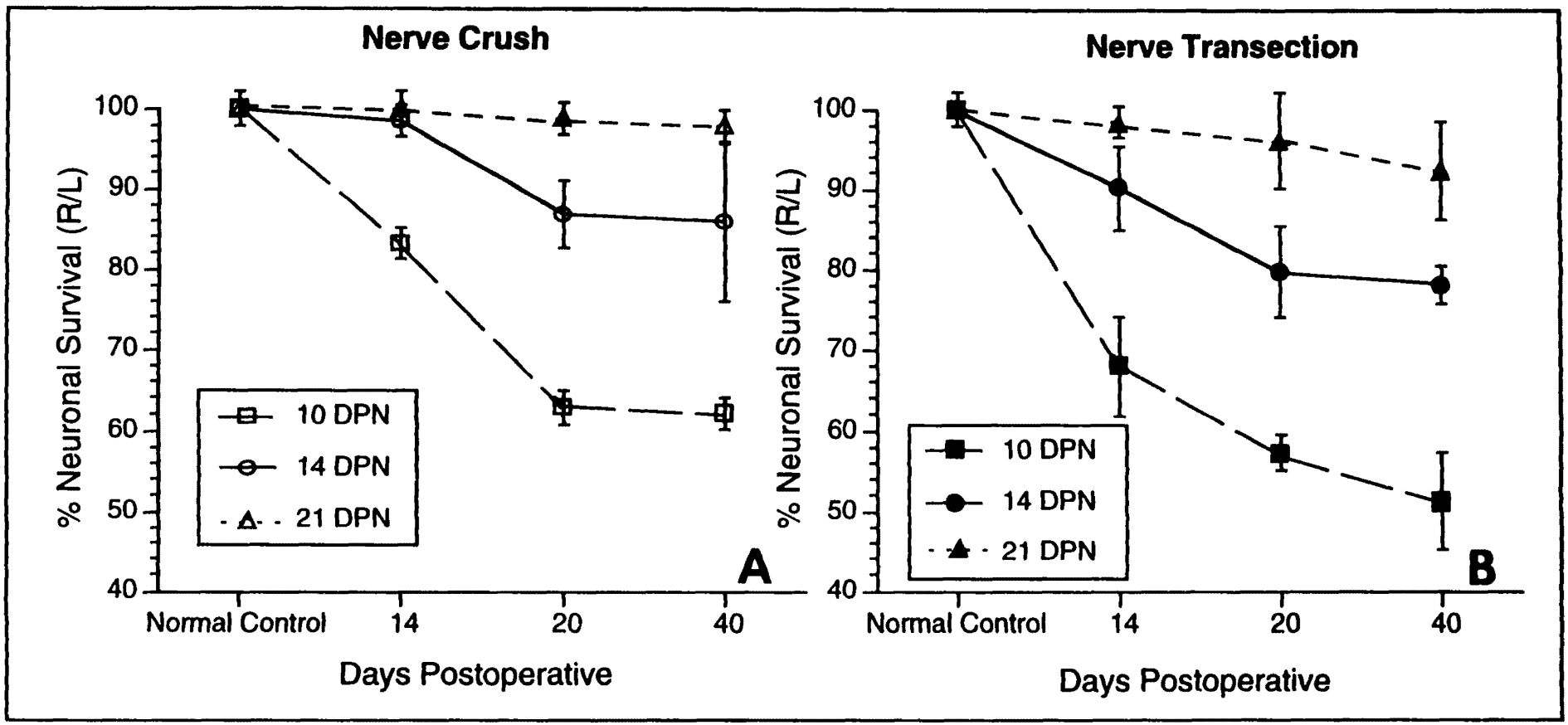
after nerve transection in the 10 dpn rats (Figs. 7B,C). In contrast, the modest increase in α CGRP mRNA detected initially after nerve crush, declined considerably to below normal levels of the intact nuclei at 6 and 18 dpo (Figs. 7D,E).

The integrated density measurements for GAPDH bands (Fig. 7F) were similar for the noninjured and injured hypoglossal nuclei.

Neuronal Survival

Neuronal cell loss occurred in the 10 and 14 dpn rats after both types of nerve injury, but not in the 21 dpn rats (Figs. 8A,B). The 10 dpn rats exhibited the earliest neuronal cell loss at 14 dpo after both types of nerve injury (Figs. 8A,B). Neuronal cell loss was completed in the 10 and 14 dpn rats by 20 dpo (Figs. 8A,B). The frequency of neuronal cell loss was not dependent upon the type of injury for the 10 and 14 dpn rats (Figs. 8A,B). However, cell counts of neuronal survival seen in tissue at 40 dpo suggest that the four day maturation that occurs from 10 to 14 dpn resulted in the rescue of 23-27% of the neuronal population after both types of nerve injury (CR = 23%; TR=27%; Figs. 8A,B).

Figure 8. Frequency of neuronal survival in hypoglossal nuclei after unilateral nerve crush (A) or transection (B). Means (± SEM) are reported. Error bars are sometimes smaller than symbols and therefore are not always visible.


Nerve Crush
% Neuronal Survival (R/L)
100
90
80
70
60
50
40
10 DPN
14 DPN
21 DPN
A
Normal Control
14
20
40
Days Postoperative
Nerve Transection
% Neuronal Survival (R/L)
100
90
80
70
60
50
40
10 DPN
14 DPN
21 DPN
B
Normal Control
14
20
40
Days Postoperative

HRP Reinnervation Assay

Control Observations

An average of 77% [+1.8] of neurons in both hypoglossal nuclei of unoperated rats and the contralateral nucleus of experimental animals contained HRP reaction product after bilateral injections of the protein into the tongue musculature. This mean percentage is in agreement with previous studies of HRP labeling in hypoglossal nuclei (Hall, 1988, Borke et al., 1993) and allows for an interneuron population that does not label after tongue injections of HRP (Boone and Aldes, 1984a).

Nerve Injury Observations

Complete reinnervation of the tongue musculature occurred in all three age groups of rats in which the hypoglossal nerve was crushed (Figs. 9A & 10A,B,C). However, complete reinnervation was achieved earlier in the oldest rats: 21 dpn rats at 20 dpo (Figs. 9A & 10C) versus the 10 and 14 dpn rats at 40 dpo (Figs. 9A & 10A,B). At the earliest postoperative time examined (14 dpo), fewer axons had reached the tongue after nerve transection (Figs. 9B & 10D, E, F) than after nerve crush (Fig. 9A).

Figure 9. Frequency of HRP-labeled neurons in hypoglossal nuclei after unilateral nerve crush (A) or transection (B). Means (± SEM) are reported. Error bars are sometimes smaller than symbols and therefore are not always visible.

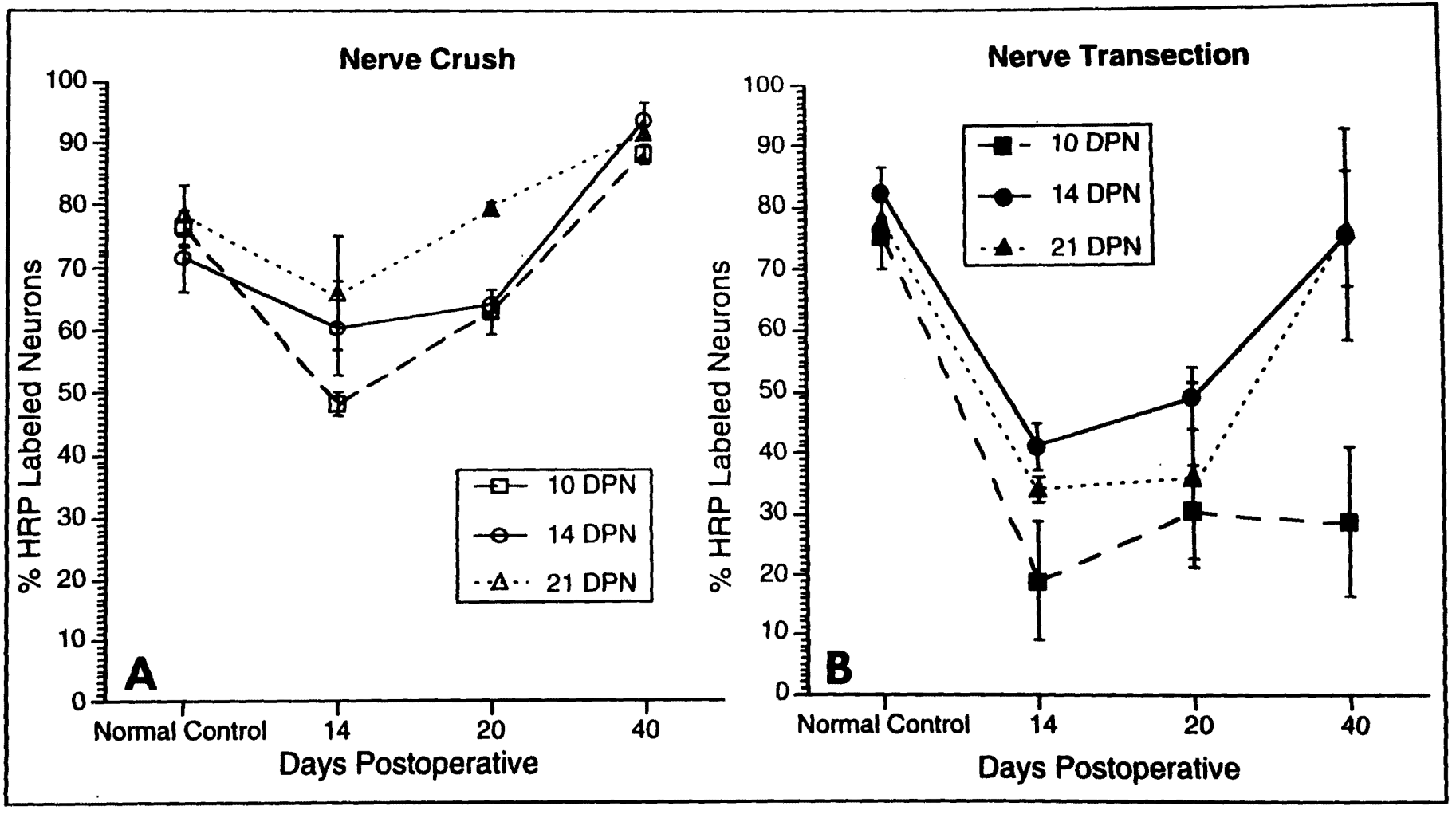
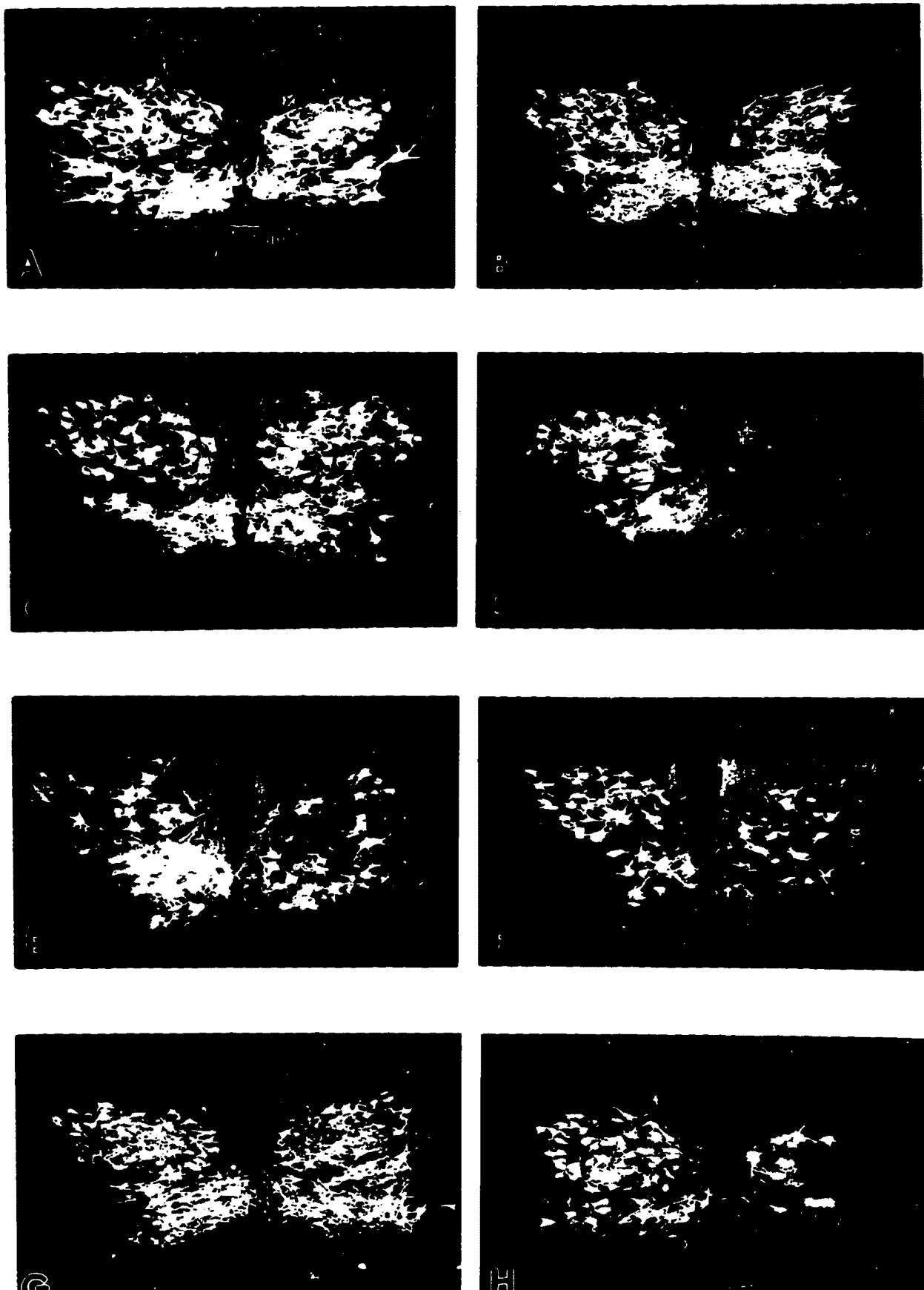

Nerve Crush
% HRP Labeled Neurons
100
90
80
70
60
50
40
30
20
10
0
10 DPN
14 DPN
21 DPN
A
Normal Control
14
20
40
Days Postoperative
Nerve Transection
% HRP Labeled Neurons
10 DPN
14 DPN
21 DPN
B
Normal Control
14
20
40
Days Postoperative

Figure 10. Dark-field micrographs of retrograde labeling of neurons in hypoglossal nuclei from bilateral tongue injections with horseradish peroxidase after unilateral hypoglossal nerve crush or transection in 10 and 21 dpn rats A: 10+40 CR; B: 14+40 CR; C: 21+20 CR; D: 10+14 TR; E: 14+14 TR; F: 21+14 TR; G: 14+40 TR; H: 10+40 TR. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.


A
G
H

Initially, the incidence of HRP-labeled cells was not directly related to the age of the rats at the time of transection; the most sizable population of parent neurons containing HRP reaction was found in the 14 dpn rats (Fig. 8B & compare Figs. 10E & 10D,F). However, complete reinnervation occurred at the same postoperative time (40 dpo) after nerve transection for both the 14 dpn (Figs. 9B & 10G) and 21 dpn rats (Fig. 9B), whereas only 30% of hypoglossal neurons in the 10 dpn nerve-transected rats had achieved reinnervation by 40 dpo (Figs. 9B & 10H).

Discussion

This study examined the effects of postnatal maturation on α CGRP mRNA and its peptide in motoneurons and whether postnatal age altered the expression of CGRP after different types of hypoglossal nerve injury. In addition, neuropeptide alterations were correlated with target reinnervation and neuronal survival.

The major findings of the current work were that: 1) during the postnatal interval when high, apparently equivalent, levels of the peptide were expressed in intact hypoglossal nuclei, axonal injury resulted in a differential regulation of CGRP; 2) α CGRP mRNA was upregulated soon after each nerve injury in both age groups, but subsequent elevations in gene expression were limited to the 21 dpn experimental rats; and 3) in the

21 dpn experimental rats, when patterns of CGRP upregulation mimicked those reported for adult motoneurons, neuronal survival and reinnervation were maximized.

Postnatal Development of CGRP in Hypoglossal Neurons

Although a transient up-regulation and gradual reduction in CGRP-IR in hypoglossal motoneurons during development has been reported (Kubota et al., 1988; Hares & Foster, 1991), no study has examined the temporal correspondence between postnatal changes in the level of α CGRP mRNA expression and translation to its peptide in cranial motoneurons. The current results on the ontogeny of CGRP expression differed from those of earlier reports that CGRP-IR was maximal at 5-6 dpn (Kubota et al., 1988; Hares & Foster, 1991) and disappeared at 28 dpn (Kubota et al., 1988). Utilization of a more sensitive method for immunodetection of CGRP (avidin-biotin Elite method vs indirect immunofluorescence) in the current work could explain the differences in the timing of the postnatal expression of CGRP in hypoglossal motoneurons. The validity of this reasoning is strengthened by the report of Morara et al. (1995) on the postnatal up-regulation of CGRP-IR in the inferior olivary nucleus in the medulla using the avidin-biotin Elite method. These authors showed a difference in peak times and duration of

CGRP-IR compared to that obtained in the same nucleus by indirect immunofluorescence (Kubota et al., 1988).

The current data provide temporal correspondence of maximal levels of peptide being generated during the middle of the second postnatal week corresponding to the period of massive expansion of tongue innervation (Li & Dahlström, 1992). This is also the time when CGRP is initially detected in motoneuron terminals (Matteoli et al., 1990; Li and Dahlström, 1992). The lack of a decrease in peptide levels at 3-4 weeks postnatal, coincident with the time when the adult CGRP response to nerve injury can first be elicited, might seem inconsistent. It is known that at the time when supernumerary synapses are pruned at the target musculature, motor end plates assume their adult form, but CGRP-IR is still abundant in these motor end plates near the end of the third postnatal week (Li & Dahlström, 1992, 1993). A reasonable premise is that CGRP levels in the hypoglossal nucleus would persist until the fourth and fifth postnatal weeks when the proportion of motoneurons containing CGRP is decreased by one-half and the number of terminals as well as peptide content of the motoneurons are reduced (Matteoli et al., 1990; Li and Dahlström, 1992, 1993).

Response of CGRP to Hypoglossal Nerve Injury during Postnatal Development

While postnatal rats examined in this study expressed comparable levels of CGRP in the intact hypoglossal nucleus, there was an age-dependent progression in CGRP expression in injured motoneurons. Changes in CGRP-IR in the 21 dpn rats mimicked results observed in the adult after both types of injury: crush and transection (Borke et al., 1993). Levels of CGRP were up-regulated until 20 dpo when reinnervation was complete following nerve crush. A biphasic response followed nerve transection that was similar to that observed in the adult (Borke et al., 1993, Grothe, 1993) and consisted of a peak in CGRP-IR at 3 dpo, a decrease to normal levels at 7 dpo, and a second increase at 20 dpo.

The changes in peptide expression in injured hypoglossal motoneurons of 21 dpn rats were preceded by alterations in α CGRP mRNA expression after both types of injury. The time course and pattern of α CGRP mRNA expression in the 21 dpn rats after nerve crush corresponded to those changes reported for adult facial motoneurons after nerve crush (Saika et al., 1991a). In addition, there was close agreement between the ratios of normalized volume densities of α CGRP mRNA in injured/noninjured hypoglossal nuclei after nerve crush and normalized densitometric ratios of CGRP mRNA in injured/noninjured spinal motoneurons after the same type of injury in adult rats (Sala et al., 1995).

The temporal progression of the biphasic increases in α CGRP mRNA after transection of the nerve also agreed with those findings in the adult rat after the same type of injury to the facial nerve (Haas et al., 1990; Dumoulin et al., 1991). The lack of finding a transient reduction in α CGRP mRNA below intact, normal levels reported recently in adult hypoglossal motoneurons (Sarasa et al., 1996) could be age-related or due to differences in postoperative timing or injury paradigms.

For the 10 and 21 day old rats, both types of injury induced an initial, rapid upregulation of α CGRP mRNA. However, nerve transection produced a greater increase in CGRP gene expression than nerve crush. Both injuries isolated the motoneuron from its target. Numerous changes can influence the magnitude of the early transcriptional events after different types of nerve injury: mechanical (Lieberman, 1971, 1974), chemical (LeBlanc and Poduslo, 1990, Knyihar-Csillik et al., 1992, Madore et al., 1994, Venezie et al., 1995), ionic (Meiri et al., 1981), electrophysiological (Herdegen et al.,1993), and/or cellular (Scherer et al., 1994, Venezie et al., 1995) events.

The question arises as to why the specific patterns of subsequent increases in CGRP expression induced in the 21 dpn nerve-injured rats at later postoperative times were not also seen in tissues from 10 dpn rats. At the time of injury, 10 dpn hypoglossal motoneurons are undergoing crucial target-dependent growth and differentiation. The generalized hypothesis from which evidence has recently emerged is that postsynaptic

muscle cells provide factors that are transported retrogradely in the motoneuron and influence the growth, differentiation, phenotypic traits and survival of the developing motoneurons (Patterson & Nawa, 1993). Only later do motoneurons respond to those factors provided by the local environment around the motoneuron and its processes (Lowrie & Vrbová, 1992).

The presence of low affinity nerve growth factor receptor (LNGFR) in immature motoneurons is consistent with the postnatal interval when neurotrophic support is requisite from the target (Yan et al., 1988; Ernfors, et al., 1989). At least four members of the neurotrophin family of basic proteins can bind to LNGFR, namely, NGF, BDNF, NT-3 and NT-4/5 (Barde, 1989; Glass & Yancopoulos, 1993). The current investigators found that LNGFR-IR was abundant on hypoglossal motoneurons and their processes at 3, 7 and 10 dpn, declined to modest levels at 14 dpn, and then became virtually absent from motoneurons by 21 dpn (see Chapter 4). Loss of LNGFR signals the postnatal time when the motoneuron transitions from target dependence to relative independence (Chiu et al., 1993). It may be of considerable relevance that axonal transection in rats during the first two postnatal weeks, produced rapid apoptosis of Schwann cells but the same injury resulted in negligible Schwann cell loss in 25 dpn rats (Trachtenberg & Thompson, 1996). These findings underscored the trophic dependence of axon-Schwann cell interaction or an axon-derived trophic factor during this postnatal interval (Trachtenberg & Thompson, 1996). Furthermore, this evidence implies that Schwann cells would

proliferate and act as a substitute target in the 21 dpn rats in response to nerve injury but are less likely to be serve as effectively as the target replacement in the 10 dpn rats. The fact that NGF mRNA rises rapidly in Schwann cells in the distal denervated nerve segment after nerve injury in adult rats, and BDNF mRNA begins to become increased at 3 dpo and continues to rise for 2 weeks emphasizes that neurotrophic factors change in a time-dependent manner in response to nerve injury (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993; Friedman et al., 1995a). This sequencing of neurotrophic changes may be related to the timing of CGRP up-regulation. In fact, there is some evidence to suggest that CGRP expression is at least in part, influenced by factors that are produced in higher than normal amounts in damaged nerves (Sala et al., 1995).

Motoneuron Cell Loss/Survival

The current results confirmed earlier evidence that injury to hypoglossal motoneurons during the first two postnatal weeks resulted in considerable neuronal cell loss that was age-related (Snider & Thanedar, 1989) and not dependent on the type of nerve injury (Borke, 1982). A new finding which could be of benefit in designing studies investigating neuronal cell death was that neuronal cell loss took place in less than three weeks in 10 and 14 dpn nerve-injured rats.

In contrast, neither injury resulted in neuronal loss in the 21 dpn rats after either type of injury, although cell death had been the outcome in another study of the same age rats (Snider & Thanedar, 1989). This inconsistency may be due to the fact that the injury prevented reinnervation (Snider & Thanedar, 1989). Since cell loss was reported at later survival periods than investigated in the current study, an alternate possibility is that with additional time some cell loss may have ensued in our 21 dpn injury paradigms. The fact that complete reinnervation of the tongue musculature occurred earlier in the 21 dpn nerve-injured rats than in adults, however argues against this idea.

Reinnervation of Tongue Musculature

Very few hypoglossal motoneurons with transected axons reinnervated the tongue musculature in the 10 dpn rats. This finding was in contrast with what occurred after nerve crush in the 10 dpn rats and after all injury paradigms in the 14 and 21 dpn rats. In all these cases, complete reinnervation of the tongue musculature from surviving motoneurons was established. A possible explanation for the failure of the 10 dpn nerve-transected neurons to successfully reinnervate the target could be related to differences in Schwann cell proliferation after the two types of injury. As mentioned earlier, nerve transection in rats from 0-14 dpn resulted in rapid apoptosis of Schwann cells (Trachtenberg and Thompson, 1996). Whether comparable Schwann cell death also occurs

after nerve crush in rats of the same ages is unknown. The fact that the initial timing and intensities of Schwann cell proliferation is similar after the two injuries in adult rats (Scherer et al., 1994) emphasizes the likelihood of this possibility. Since Schwann cell proliferation persists longer after nerve crush in adults (Scherer et al., 1994), an alternative idea is that those Schwann cells that survive a nerve crush, as opposed to a nerve transection, are better able to carry out reparative functions such as axonal guidance (Keynes, 1987, Johnson et al., 1988, Zhao et al., 1992, Anton et al., 1994, Torigoe et al., 1996).

Conclusion

The present evidence indicates that profiles of CGRP expression after different nerve injury paradigms during postnatal life are dependent on the state of interaction of the motoneuron axon with its local environment and its target musculature. When the response to nerve injury parallels that elicited in adult hypoglossal motoneurons, neuronal survival/reinnervation are the likely outcomes.

CHAPTER 3.

Choline Acetyltransferase Immunoreactivity in Cranial Motoneurons After Hypoglossal Nerve Injury During Postnatal Development

Materials and Methods

Animal Population

One hundred and seventy-two Sprague-Dawley rats of both sexes were used for this investigation. Hypoglossal nerve crush or nerve transection was performed in four animals of each postnatal age group (10, 14, and 21 dpn) for each of the six different survival times (1,3,7,14,20 and 40 dpo). ChAT immunocytochemistry was utilized for the experimental and control series. A normal developmental series consisted of four rats at seven different postnatal ages (3, 7,10, 14, 17, 21, and 30 dpn) for a total of 28 rats.

Surgical Procedures

Early postnatal rats are insensitive to small dosages of most injected anesthetics (Borke, 1982). Therefore, 10 and 14 dpn rats were

anesthetized with ether. For rats 21 dpn or older either 7% chloral hydrate (0.5 ml per 100 g) or a ketamine/rompun combination (ketamine [50 mg/kg], rompun [10 mg/kg]) was used as the anesthetic. The nerve injury was performed on the right hypoglossal nerve proximal to its bifurcation into medial and lateral branches. The nerve injuries consisted of either a nerve crush for three minutes using a self-locking needle holder with smooth jaws or a nerve transection with apposition of the cut ends.

Following surgery the pups were returned to their dams. Pup weights were recorded daily. Supplemental gavage feeding and soft foods were provided to ensure appropriate weight gain in the young animals. Twenty-one day and older rats were kept to a standard *ad libitum* diet of pellets and water.

Immunocytochemistry

The rats were reanesthetized at the appropriate postoperative times and perfused transcardially with a fixative of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were removed and postfixed for two hours (h). The medullas were infiltrated overnight in 30% sucrose in 0.1 M phosphate buffer at 4° C, freeze-thawed in isopentane and cut into 40 μm transverse sections on a freezing microtome. Sections of the medulla through the rostro-caudal extent of the hypoglossal nucleus were

collected in Tris buffered saline (TBS; pH 7.6). Free-floating sections were used for ChAT immunocytochemistry.

Sections were pretreated with 10% normal goat serum (NGS; Cappel) in TBS for 1 h and incubated for 48 h at 4° C in a monoclonal antibody from rat-mouse hybrid cells directed against ChAT (1:10; Boehringer Mannheim) in TBS with 20% NGS and 2% bovine serum albumin. Sections were washed in TBS and incubated for 1 h at room temperature in biotinylated goat anti-rat IgG (1:100; Vector) in TBS containing 1.5% NGS. After a TBS wash, the sections were incubated at room temperature in ABC Standard complex (1:50; Vector) for 1 hour. Immunoreactivity was visualized with 3-3'diaminobenzidine (DAB; 0.06%) and H_2O_2 (0.005%) in 0.1 M phosphate buffer. Sections were mounted on glass slides, air dried, and dehydrated, cleared, and coverslipped with Permount. Controls for the immunocytochemical procedures were processed by omitting the specific primary antiserum or by using antisera preabsorbed with an excess of the immunogen. No positive staining was detected after either treatment.

Immunocytochemical Analysis

The levels of ChAT-IR were assessed quantitatively with the aid of a computerized densitometry analysis system using NIH Image software (Version 1.54) linked to an Olympus microscope. For each case, four

sections of the hypoglossal nucleus were randomly selected through the center of the hypoglossal nucleus for image analysis. Each section was digitized at a final magnification of 40 x using a MTI CCD 72 camera and a Scion LGF image grabber card in a Macintosh Quadra 800 (Apple Computer, USA). The gray scale used ranged from 0 for white to 255 for black. The final background level was determined from an unlabeled region of the adjacent reticular formation. Using a box measuring 50 x 50 pixels, mean pixel intensities were determined and averaged from three different areas of the nucleus (neurons plus neuropil) on the right and the left sides. The mean values from four cases/developmental group were converted to right/left (R/L) ratios and mean ratio values were used to determine the percent change in immunocytochemical staining intensity for the antigen. These values from each injured treatment group were then compared to the data for the non- injured group for each age group by a one way ANOVA. The Student Newman Keuls test was applied *post hoc* to inspect differences between means when F values from ANOVA produced $p < 0.05$ (Snedecor and Cochran, 1980). All computations were performed with the Graph Pad Instat statistical package.

Results

ChAT Immunoreactivity

Noninjured Hypoglossal Nuclei

Hypoglossal motoneurons were immunoreactive for ChAT at all postnatal ages of this study [3-30 dpn] (Fig. 11A-F). Slight to moderate ChAT-IR was seen in hypoglossal neuronal cell bodies of 3 and 7 dpn rats (Fig. 11A,B). The mean pixel intensity of ChAT-IR nearly tripled from 7 to 10 dpn (Fig. 12 [$p<.001$]). This intense level of ChAT-IR was maintained in hypoglossal neurons of 10-30 dpn rats (Fig. 12). However, ChAT staining in the hypoglossal neuropil increased progressively from 17-30 dpn (Fig. 11C,D,E,F). The specificity of ChAT-IR was confirmed by additional bilateral labeling confined to the dorsal motor nucleus of the vagus (Fig. 11 A-F) and the nucleus ambiguus.

Figure 11. ChAT-IR in the hypoglossal nuclei of normal, noninjured rats: A: 3 dpn; B: 7 dpn; C: 10 dpn; D: 14 dpn; E: 21 dpn; F: 30 dpn.

Developmental Progression of CHAT

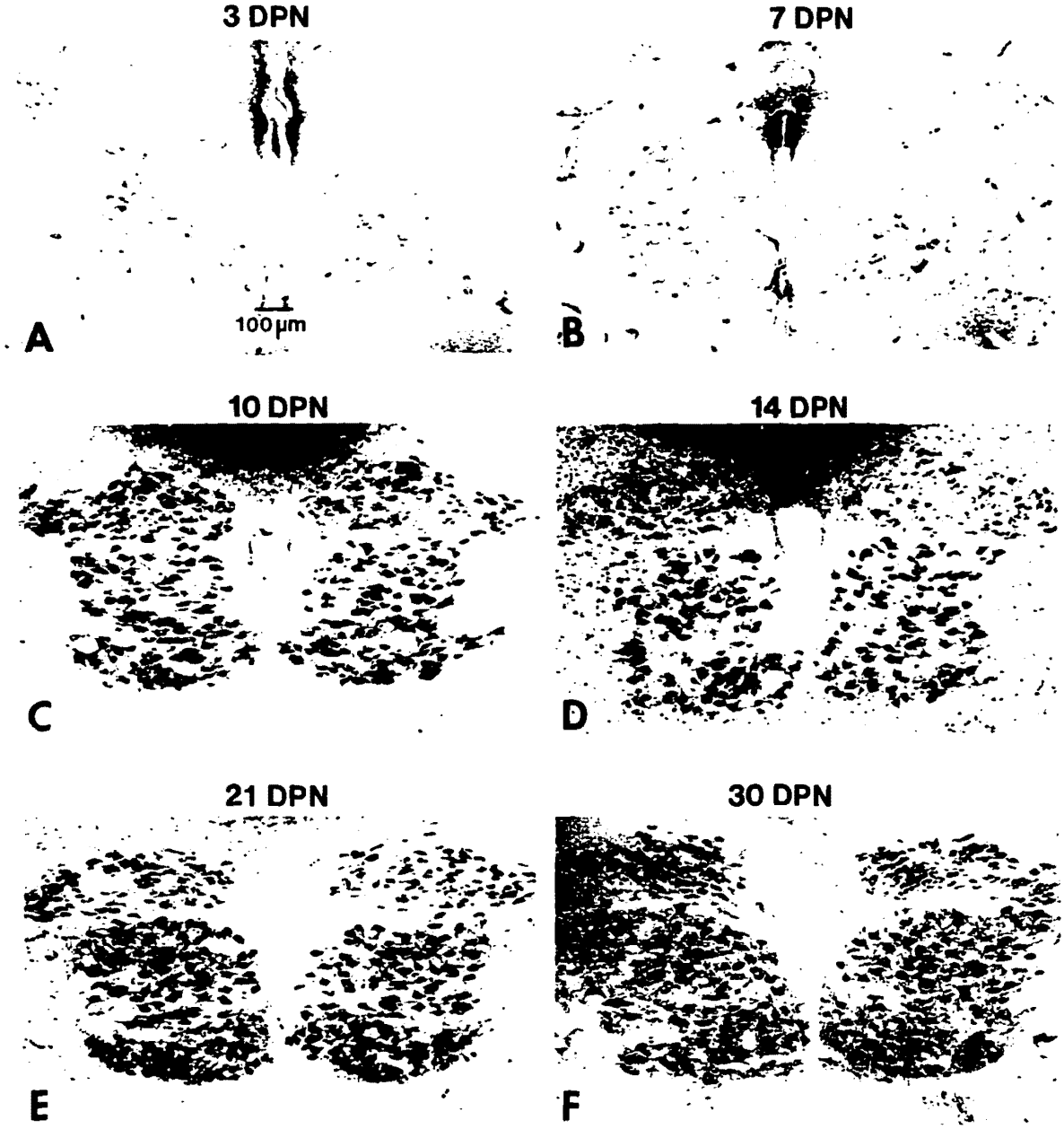
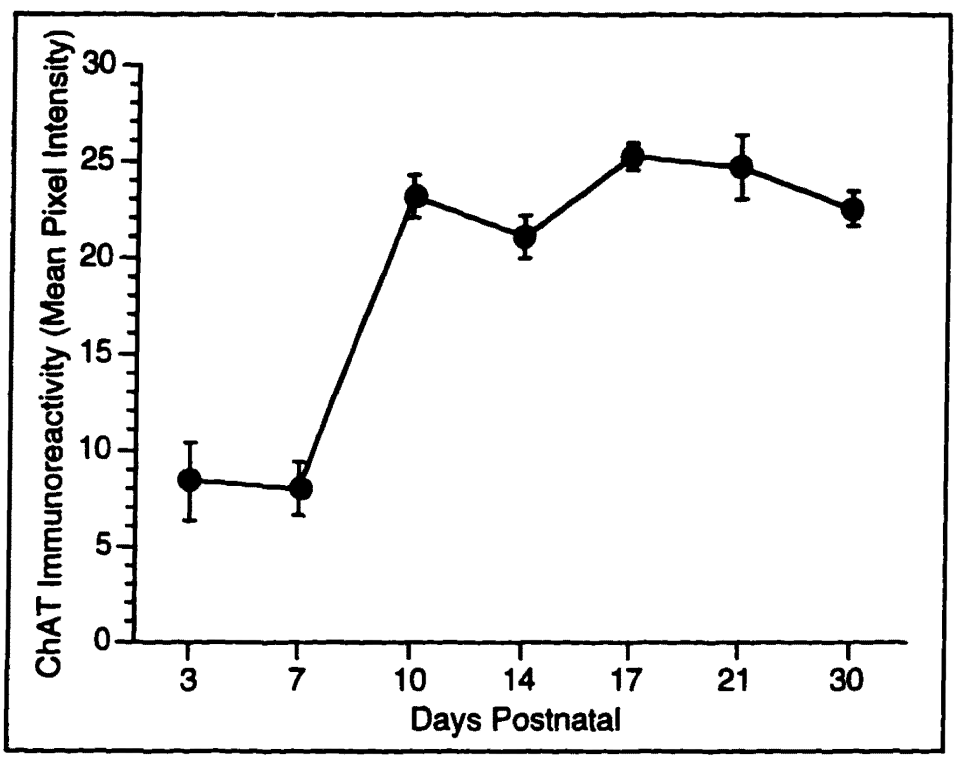


Figure 12. Change in mean pixel intensity of ChAT-IR in hypoglossal nuclei during postnatal development. Means (± SEM) are reported.


ChAT Immunoreactivity (Mean Pixel Intensity)
30
25
20
15
10
5
0
3
7
10
14
17
21
30
Days Postnatal

Injured Hypoglossal Nuclei

Nerve Crush

The timing and magnitude of changes in ChAT-IR were comparable for all 3 ages at all postoperative survival periods (Fig. 13A). Little to no decrease in ChAT-IR was detected at 1 dpo in 10, 14, or 21 dpn rats after nerve crush (Fig. 13A). Maximal reduction of ChAT-IR was found at 3 dpo for all age groups (Fig. 13A; [$p<0.001$]). At this time, very few hypoglossal neurons were immunoreactive for ChAT (Figs. 14A, 15A, & 16A). For all 3 ages, at 7 dpo, there was a comparable increase in the number of ChAT-IR neurons in hypoglossal nuclei ipsilateral to the nerve crush (Fig. 13A, [$p<0.001$] & Figs. 14C, 15C, 16C). The progressive increase in the number of ChAT-IR neurons continued so that by 14 dpo, ChAT staining had returned to all injured hypoglossal neurons as well as the neuropil (Fig. 13A, [$p<0.001$] & Figs. 14E, 15E, 16E). In fact, ChAT staining seemed more intense in the hypoglossal nucleus of 14 and 21 dpn nerve crush rats on the injured side compared to the noninjured contralateral nucleus at 14, 20, and 40 dpo. This increase was statistically significant in the 21 dpn rats at 14 and 20 dpo [$p<0.01$] (Fig. 13A & Figs. 15E, 15G, 16E, 16G).

Figure 13. Percent change in staining intensity of ChAT-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (A) or transection (B). Means (± SEM) are reported. Error bars are sometimes smaller than symbols and therefore are not always visible.

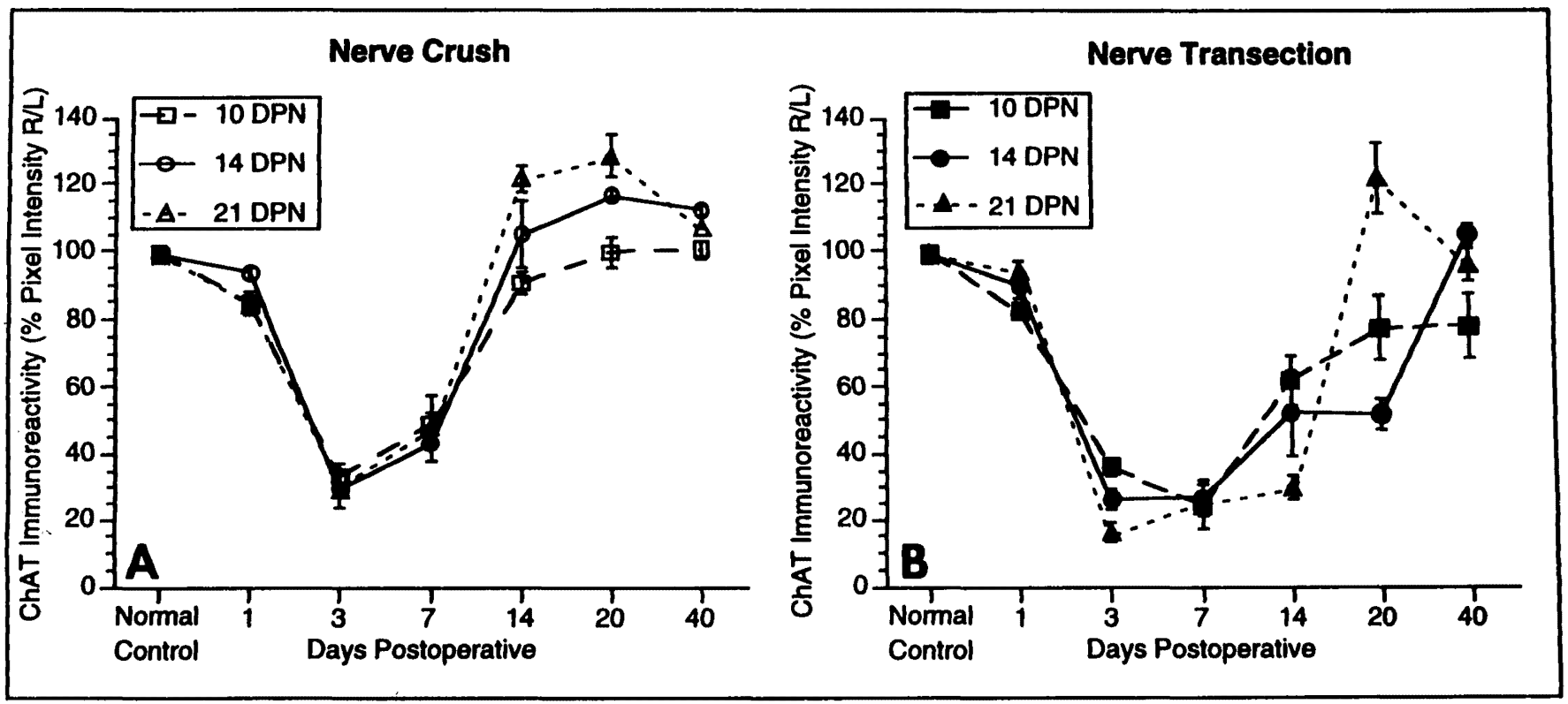

Nerve Crush
Nerve Transection
10 DPN
14 DPN
21 DPN
ChAT Immunoreactivity (% Pixel Intensity R/L)
0
20
40
60
80
100
120
140
A
B
Normal Control
1
3
7
14
20
40
Days Postoperative

Figure 14. CHAT-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 10 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.

10DPN-CHAT

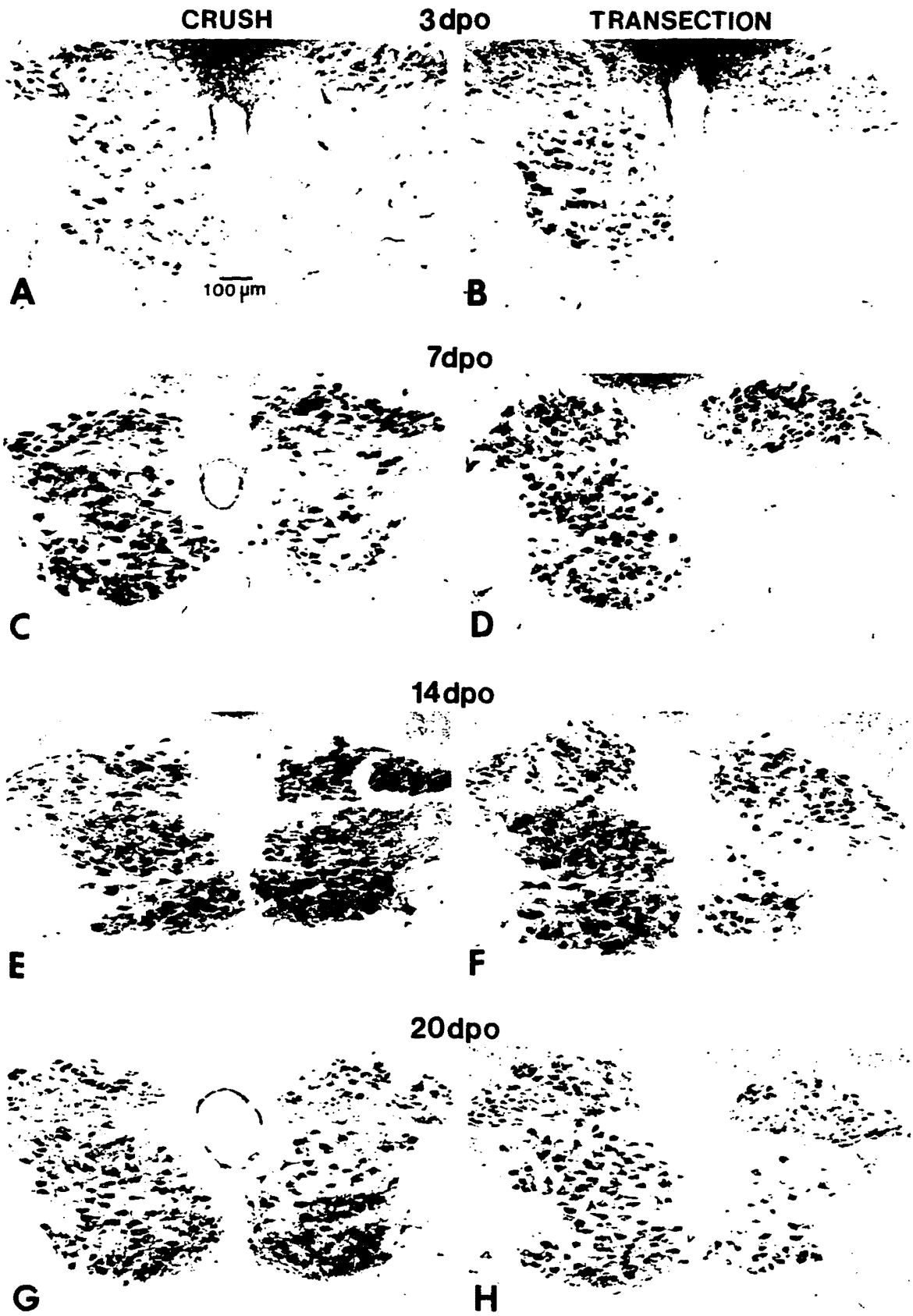


Figure 15. ChAT-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 14 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.

14 DPN-CHAT

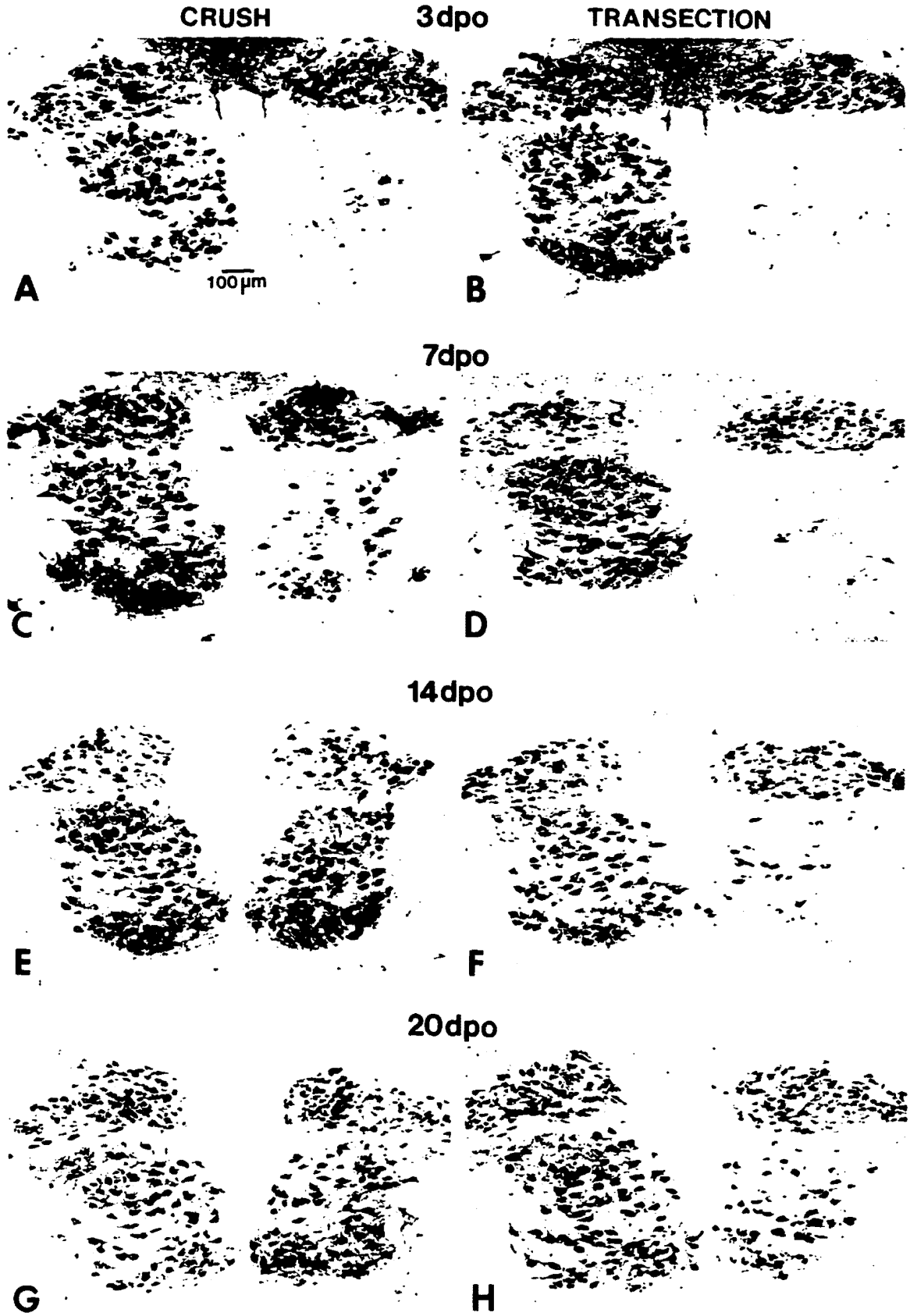
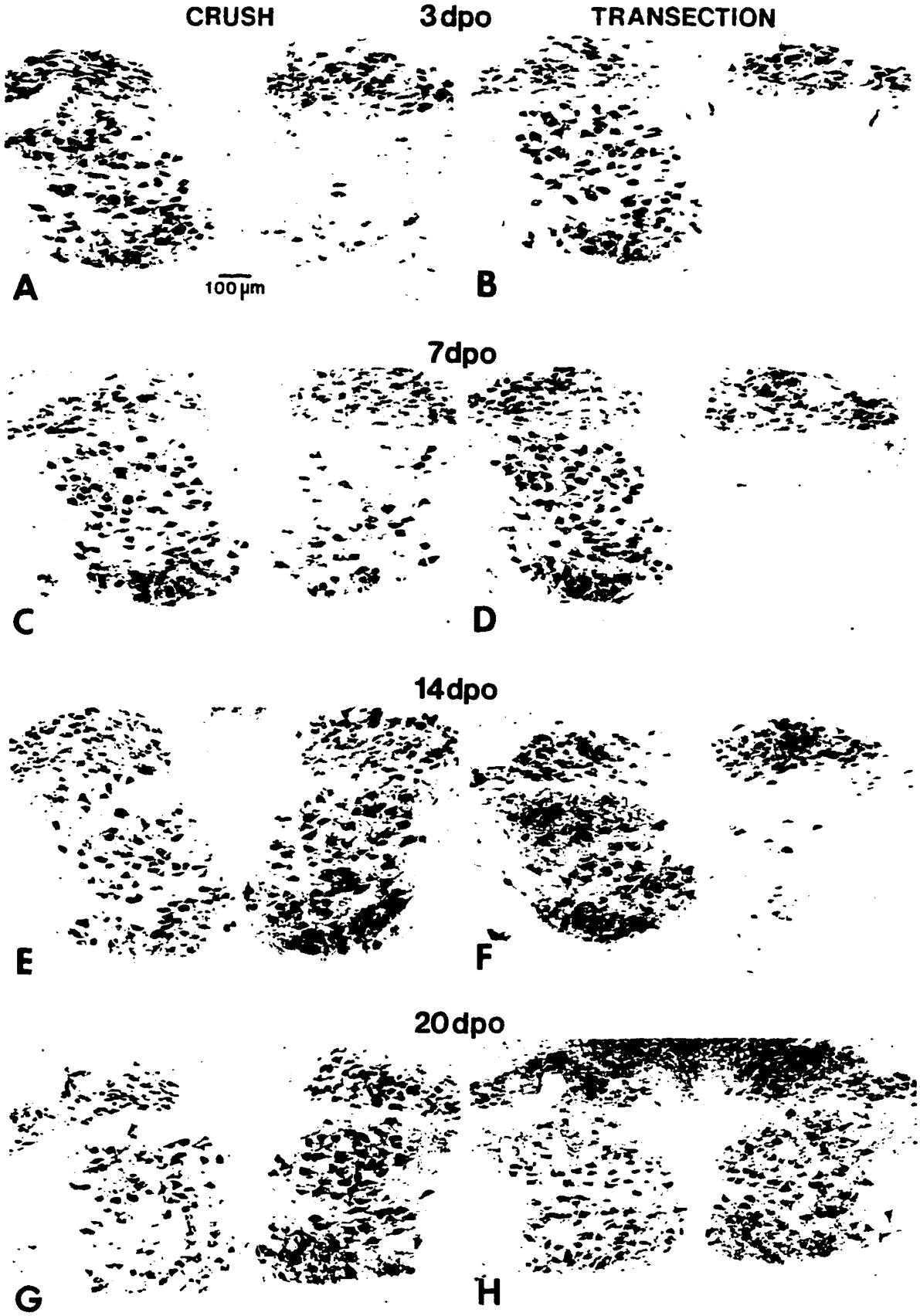


Figure 16. ChAT-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 21 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.

21 DPN-CHAT



Nerve Transection

As after nerve crush, there was no reduction in ChAT staining at 1 dpo after nerve transection in all 3 experimental age groups, but marked depletion of ChAT-IR occurred in the affected nucleus at 3 dpo (Figs. 14B, 15B, 16B; [$p<0.001$]). The amount of the decrease at 3 dpo in the 10 and 14 dpn rats was comparable for the two types of injuries (Figs. 13A & 13B). However, for the 21 dpn rats, there was a greater loss of ChAT-IR after transection than after crush (Figs 13A, 13B, 16A & 16B [($p<.01$]). Unlike after nerve crush, transection-induced loss of ChAT staining was maintained until 7 dpo for all ages (Fig. 13B; [$p<0.001$] & Figs. 14D, 15D, 16D). Further persistence of reduced levels of ChAT-IR was related to the age of the rat at the time of nerve transection. In 10 dpn nerve-transected rats, there was a progressive increase in ChAT staining in the hypoglossal nucleus on the injured side from 14-40 dpo (Figs. 13B & 14F, 14H) but ChAT-IR in the affected nucleus never matched that level of immunoreactivity found in the contralateral, noninjured hypoglossal nucleus (14 dpo [$p<0.001$], 20 and 40 dpo [$p<0.05$]) (Fig. 13B). ChAT staining gradually reappeared from 14-40 dpo in the 14 dpn rats (Figs. 15F & 15H; 14 and 20 dpo [$p<0.001$]), so that ChAT-IR was equivalent to that of the noninjured nucleus at 40 dpo (Fig. 15B). Levels of ChAT-IR remained depressed at 14 dpo in the 21 dpn rats (Figs. 13B; [$p<0.001$] & 16F). However, by 20 dpo in the 21 dpn nerve-transected rats, ChAT-IR in the hypoglossal nucleus on the injured side was elevated over the

normal level of the contralateral noninjured nucleus (Fig. 16H; [$p<0.05$]) and declined to normal ChAT-IR by 40 dpo (Fig. 13B).

Discussion

This work details the temporal expression of ChAT within hypoglossal motoneurons during postnatal development and after peripheral nerve injury. In the normal, noninjured hypoglossal nucleus, maximal levels of ChAT-IR were detected in motoneuronal somata at 10 dpn and the intensity of enzyme staining also became prominent in the neuropil from 17-30 dpn. The changes in ChAT-IR after nerve crush or transection in 10, 14 and 21 dpn rats paralleled those reported after the same injuries to adult hypoglossal motoneurons (Borke et al., 1993) with these exceptions: 1) the extent of the initial reduction in ChAT-IR was not injury-specific for the 10 and 14 dpn rats as it was for the 21 dpn and adult rats and 2) the restoration of transection-induced depletion of ChAT-IR to the level of the normal, noninjured nucleus occurred earlier in the 21 dpn than in adult motoneurons.

Postnatal Development of ChAT in Hypoglossal Neurons

This is the first study to examine the postnatal expression of ChAT in the hypoglossal nucleus, a medullary cell group known to contain the

highest adult level of ChAT activity (Kobayashi et al., 1975). The timing of postnatal changes in ChAT-IR of hypoglossal motoneurons correlates well with the progression of ChAT expression in developing spinal motoneurons (Phelps et al., 1984; Chiu et al., 1993). ChAT-IR was initially detected as early as E13 in rat spinal motoneurons and increased by small increments throughout the remainder of embryonic life and the first postnatal week (Phelps et al., 1984). At the beginning of the second postnatal week a substantial increase in ChAT-IR occurred and spinal motoneurons attained adult levels by 3-4 weeks (Phelps et al., 1984). In another study, ChAT-IR increased in spinal motoneurons so that by 10 dpn the maximal number of neurons expressed ChAT-IR but the intensity of enzyme staining continued to increase in motoneuronal processes and neuropil until 30 dpn (Chiu et al., 1993). In the current work, intense levels of ChAT-IR were attained in hypoglossal motoneuron somata by 10 dpn. ChAT staining in the hypoglossal neuropil from 17-30 dpn paralleled the timing and pattern of distribution of postnatal changes in spinal motoneuronal ChAT-IR in the ventral horn of the rat spinal cord (Chiu et al., 1993). This progression also fits well with evidence on ChAT mRNA expression in the developing rat CNS, of which the medulla and pons contain the highest levels (Ibáñez et al., 1991). Increases are biphasic with the maximal expression of ChAT mRNA occurring in the second postnatal week, followed by a second lesser increment in enzyme expression between 3-5 weeks (Cavicchioli et al., 1991; Ibáñez et al., 1991).

In all likelihood, the majority of ChAT staining in the neuropil from 17-30 dpn in the current work was contained in fibers ending on hypoglossal motoneurons. The validity of this idea is strengthened by evidence that 1) acetylcholinergic neurons in the reticular formation provide a primary source of input to hypoglossal motoneurons (Connaughton et al., 1986) and 2) afferent synapses on hypoglossal motoneurons become more frequent and differentiate during the second and third postnatal weeks (Borke, 1982).

Response of ChAT to Hypoglossal Nerve Injury during Postnatal Development

The initial reduction in ChAT-IR was not detected until 3 dpo in the current work involving crush or transection injuries to rats of increasing postnatal ages. The onset of these changes in ChAR-IR paralleled those reported after crush, transection (Armstrong et al., 1991; Borke et al., 1993) or resection (Borke et al., 1993) of the hypoglossal nerve in adult rats. Thus, our results confirmed that the timing of the initial enzyme reduction is not injury-related and extended these findings to include age, another parameter known to affect the retrograde reaction of axotomized neurons (Lieberman, 1974). The onset of ChAT decrease seems to be regulated by a mechanism that is related to the interruption of axon-target interaction. The fact that ChAT-IR is also reduced by impeding axoplasmic transport without injuring the nerve reinforces this idea (Moix et al., 1991; Greeson et al., 1992).

The amount of ChAT reduction in the 10 and 14 dpn rats was similar for both injuries. This finding can be contrasted to the detection of smaller reductions in ChAT-IR in the 21 dpn motoneurons after nerve crush compared to nerve transection. This injury-specific decrease in ChAT-IR in 21 dpn experimental rats is the same as that noticed in adult motoneurons after the same types of nerve injury (Borke et al., 1993; Friedman et al., 1995a). Variation in the amount of axotomy-induced loss in ChAT-IR has been associated with differences in the accessibility of the proximal, surviving axons of a crushed versus a transected nerve to the supply of neurotrophins, such as BDNF and NT-4/5 provided by Schwann cells of the distal degenerating nerve (Friedman et al., 1995a). Consistent with these effects is the attenuation in the loss of ChAT after the application of exogenous neurotrophins to the proximal end of transected nerves (Chiu et al., 1994; Yan et al., 1994; Friedman et al., 1995a). Whether there is an increase in Schwann cell-produced neurotrophic factors with advancing postnatal age in rats is not known. It may be relevant that axonal injury during the first two postnatal weeks resulted in rapid apoptosis of Schwann cells but injury in 25 dpn rats did not (Trachtenberg & Thompson, 1996). On the other hand, ChAT expression has been shown to be influenced by factors released centrally as well as peripherally (Weiser et al., 1994). Differences in the ability of maturing neurons to acquire these factors or developmental differences in the dependency of the motoneurons on target and central factors, then, could explain the age differences in the extent of the ChAT reduction after the two injuries.

During the survival times examined, return of ChAT-IR to normal levels was obtained in all experimental groups except the 10 dpn nerve-transected rats. Interestingly, the surviving neurons only attained 29% reinnervation of tongue musculature by 40 days after nerve transection in the 10 dpn rats, whereas complete reinnervation was reached by 20 (21 dpn) or 40 days (10 and 14 dpn) after nerve injury for all other experimental age groups (see Chapter 2). The current findings indicate that the return of ChAT-IR to normal, noninjured levels precedes reinnervation of the tongue musculature. Whether ChAT-IR would eventually be restored at later postoperative times even without complete reinnervation, as occurs in adult hypoglossal motoneurons (Borke et al., 1993; Chen et al., 1995), remains to be determined.

Of particular interest was the fact that ChAT-IR returned to normal levels considerably earlier (20 dpo) after hypoglossal nerve transection in 21 dpn rats than after the same injury in adult rats (Armstrong et al., 1991; Borke et al., 1993; Rende et al., 1995). The resumption of normal levels of ChAT was also found earlier after facial nerve transection in 14 and 21 dpn mice compared to adults (Kou et al., 1995). These findings are not surprising since target reconnection and recovery of function occurred more rapidly in young mammals after nerve transection (Lieberman et al., 1974; Vaughan, 1990). Likewise, young motoneurons that survive axotomy and reinnervate their target demonstrate transiently, increased reflexes and polyinnervation of the musculature, as well as, an expanded dendritic field (O'Hanlon and Lowrie, 1993). In the 21 dpn nerve-

transected rats, the brief increase in ChAT in the lesioned nucleus above the level in the contralateral, noninjured nucleus may reflect an optimized interaction between the nerve and its target.

CHAPTER 4.

Expression of Low Affinity Nerve Growth Factor Receptor After Hypoglossal Nerve Injury During Postnatal Development

Material and Methods

Animal Population

Adjacent sections from selected cases from animals euthanized for the ChAT study discussed in Chapter 3 were used as the animal population for this study. Hypoglossal nerve crush or nerve transection was performed in four animals at each of three postnatal ages (10, 14, and 21 dpn) and the animals were euthanized at four different survival times (3, 7, 14, and 20 dpo). A normal developmental series consisted of four rats at seven different postnatal ages (3, 7,10, 14, 17, 21, and 30 dpn). LNGFR immunocytochemistry was utilized for the experimental and control series.

Surgical Procedures

Early postnatal rats are insensitive to small dosages of most injected anaesthetics (Borke, 1982). Therefore, 10 and 14 dpn rats were anesthetized with ether. For rats 21 dpn or older either 7% chloral hydrate (0.5 ml per 100 g) or a ketamine/rompun (ketamine [50 mg/kg], rompun [10 mg/kg]) was used as the anesthetic. The nerve injury was performed on the right hypoglossal nerve proximal to its bifurcation into medial and lateral branches. The nerve injuries consisted of either a nerve crush for three minutes using a self-locking needle holder with smooth jaws or a nerve transection with apposition of the cut ends.

Following surgery the pups were returned to their dams. Pup weights were recorded daily. Supplemental gavage feeding and soft foods were provided to ensure appropriate weight gain in the young animals. Twenty-one day and older rats were kept to a standard *ad libitum* diet of pellets and water.

Immunocytochemistry

The rats were reanesthetized at the appropriate postoperative times and perfused transcardially with a fixative of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were removed and postfixed for two h. The medulla was infiltrated overnight in 30% sucrose at 4° C,

freeze-thawed in isopentane and transverse sections were cut at 40 μm on a freezing microtome. Sections of the medulla throughout the length of the hypoglossal nucleus were collected in Tris buffered saline (TBS; pH 7.6). Adjacent free-floating sections were used for LNGFR immunoreactivity (LNGFR-IR).

Sections were pretreated with 10% NGS in TBS for 1 h and incubated 21 h at room temperature in 192 IgG, mouse anti-nerve growth factor receptor monoclonal antibody (1:20; Boehringer Mannheim) in TBS. This antibody recognizes only the non-transducing low affinity NGF receptor 192 IgG. Sections were washed in TBS and incubated for 2 h at room temperature in biotinylated horse anti-mouse IgG (1:100; Vector) in TBS containing 10% NGS. After a TBS wash, the sections were incubated at room temperature in ABC ELITE Standard complex (1:50; Vector) for 2 h. Immunoreactivity was visualized with 3-3'diaminobenzidine (DAB; 0.06%) and H_2O_2 (0.005%) in 0.1 M phosphate buffer. Sections were mounted on glass slides, air dried, and dehydrated, cleared, and coverslipped with Permount. Controls for the immunocyto-chemical procedures were processed by omitting the specific primary antiserum or by using antisera preabsorbed with an excess of the immunogen. No positive staining was detected after either treatment.

Immunocytochemical Analysis

Changes in LNGFR in tissue of intact rats of increasing postnatal ages and after the 2 types of nerve injury were examined to determine if rats of increasing postnatal age exibited alterations in the temporal expression of LNGFR in response to peripheral nerve injury.

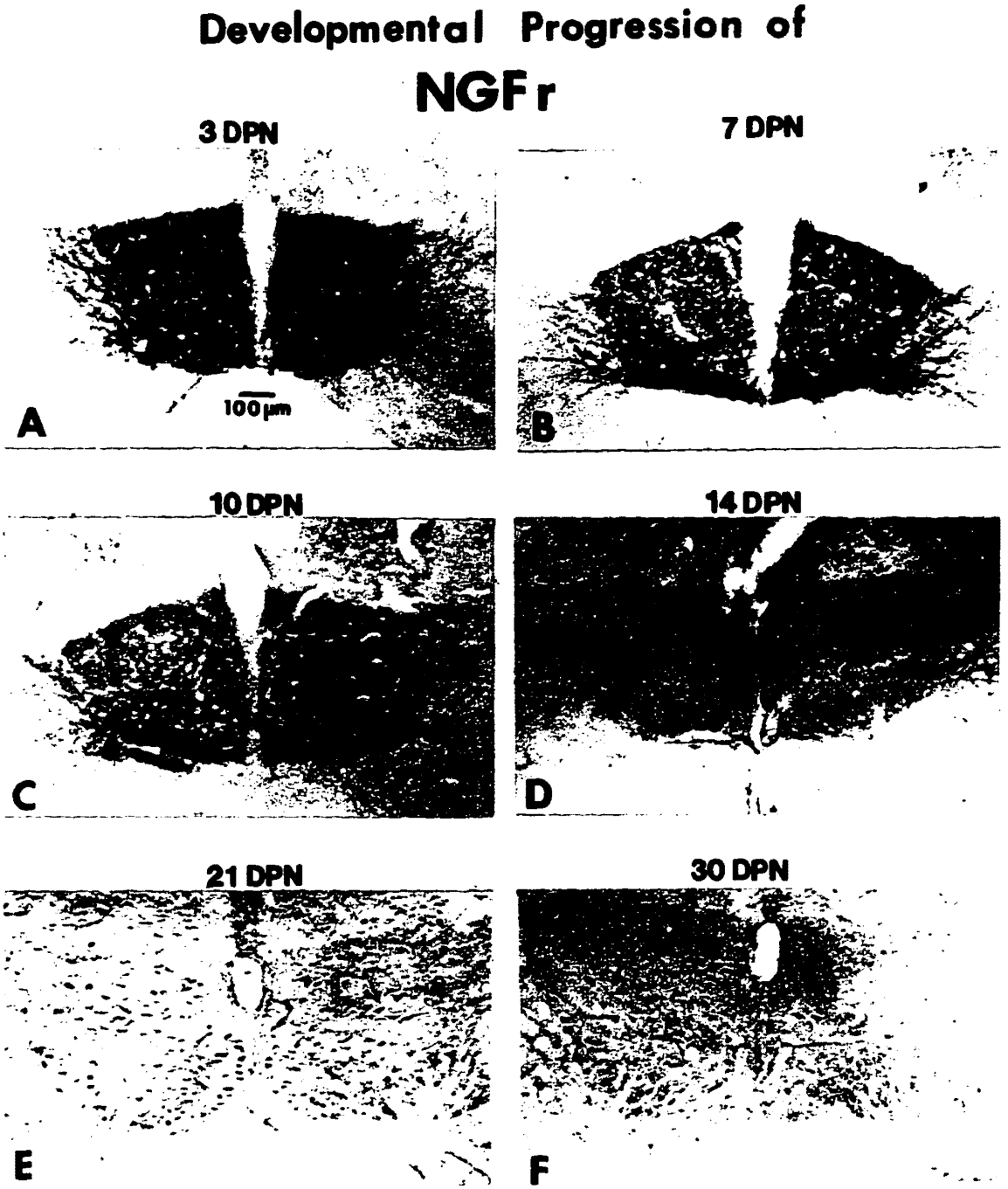
Results

LNGFR Immunoreactivity

Noninjured Hypoglossal Nuclei

Hypoglossal motoneurons exhibited decreased immunoreactivity for LNGFR with increasing postnatal age (Fig.17). Motoneurons and their processes in the hypoglossal nucleus of 3, 7, and 10 dpn rats (Fig. 17 A, B, C) demonstrated intense LNGFR-IR. This intense LNGFR staining decorated the hypoglossal dendritic processes that extended into the lateral reticular formation (Fig. 17A,B). Marked reduction in LNGFR-IR was noticed in the hypoglossal nucleus at 14 dpn (Fig. 17D). By 21-30 dpn, LNGFR-IR had disappeared from the hypoglossal nuclei (Figs. 17E,F).

Figure. 17. LNGFR-IR in the hypoglossal nuclei of normal, noninjured rats: A: 3 dpn; B: 7 dpn; C: 10 dpn; D: 14 dpn; E: 21 dpn; F: 30 dpn.


Developmental Progression of
NGFr
3 DPN
7 DPN
100 µm
A
B
10 DPN
14 DPN
C
D
21 DPN
30 DPN
E
F

Injured Hypoglossal Nuclei

Nerve Crush

At 3 dpo after nerve crush to 10 dpn rats LNGFR-IR was of similar intensity bilaterally. The level of LNGFR staining was comparable to that obtained from tissue contained in intact nuclei in 10 dpn noninjured rats (Fig. 18A compare to 17 C). However, there was an increase in LNGFR-IR in the neurons and neuropil of parent hypoglossal nuclei of 14 and 21 dpn rats at 3 dpo after nerve crush (Figs. 19A & 20A). In these rats, neurons in the contralateral, noninjured nucleus were not immunoreactive for LNGFR. The failure of 10 dpn rats to demonstrate any early elevation of LNGFR-IR over that normally detected in these young rats was the only age-rerlated difference found in LNGFR-IR after nerve crush. The increase in LNGFR-IR in the injured nucleus was maximal at 7 dpo for all 3 ages (Figs. 18C, 19C & 20 C). The staining decreased at 14 dpo and disappeared by 20 dpo in rats injured at 10, 14, and 21 dpn (Figs. 18E, 18G, 19E, 19G, 20E & 20G).

Nerve Transection

For the 10 and 14 dpn rats, nerve transection produced an increase of LNGFR-IR that was of less magnitude but of longer duration than the

Figure. 18. LNGFR-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 10 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.

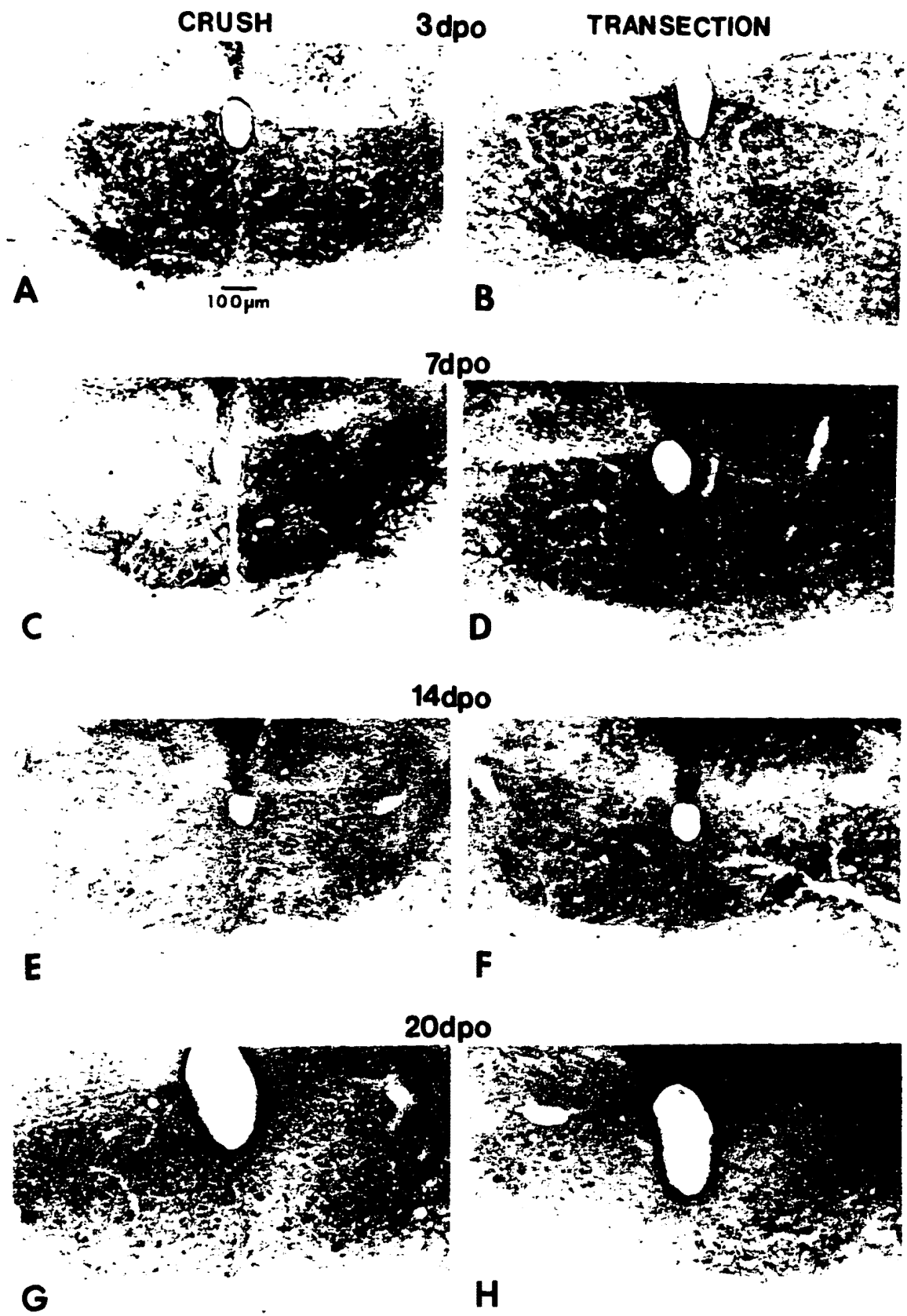

10DPN-NGFr
CRUSH
3dpo
TRANSECTION
A
100μm
B
7dpo
C
D
14dpo
E
F
20dpo
G
H

Figure. 19. LNGFR-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 14 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.

14 DPN-NGFr

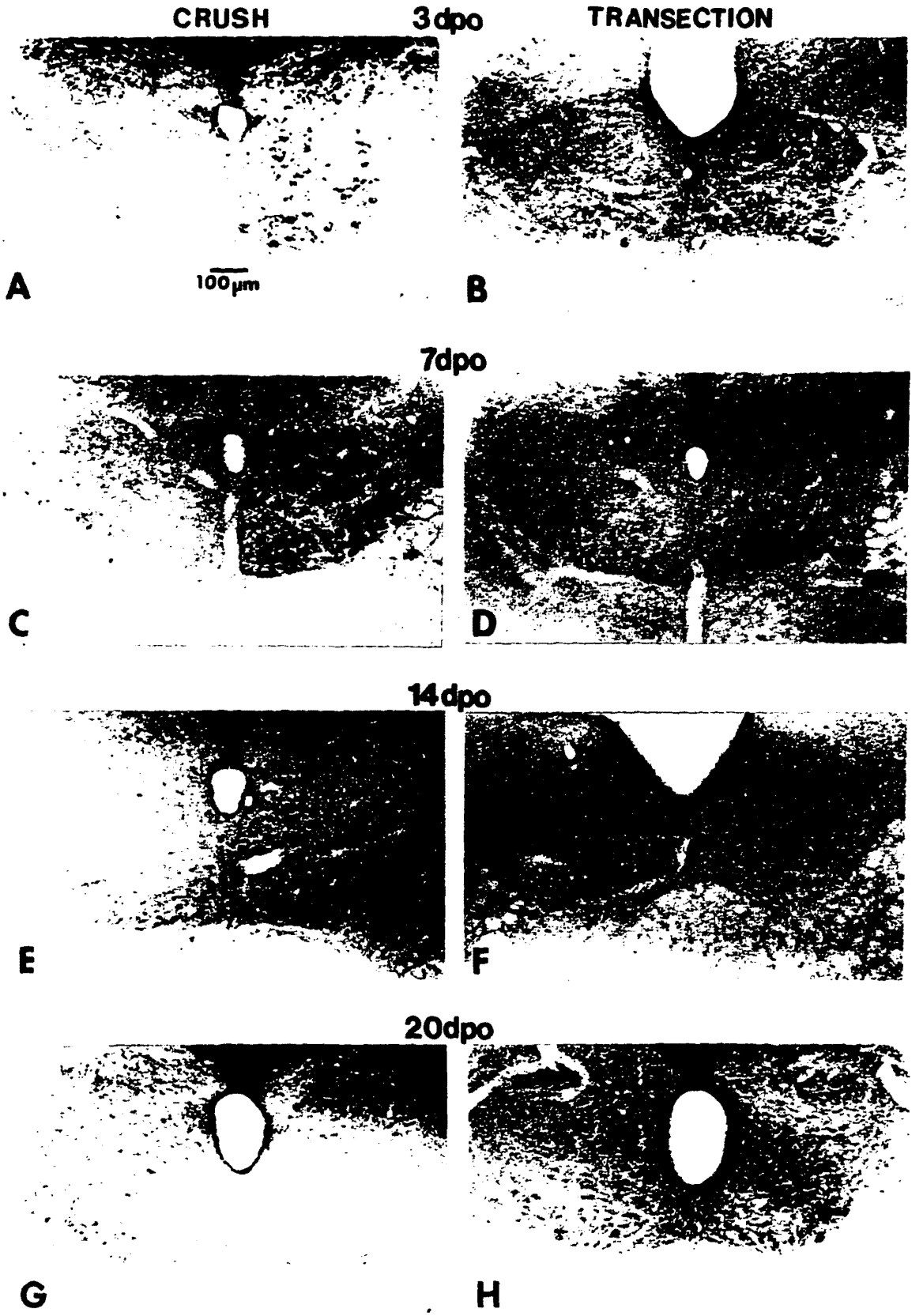
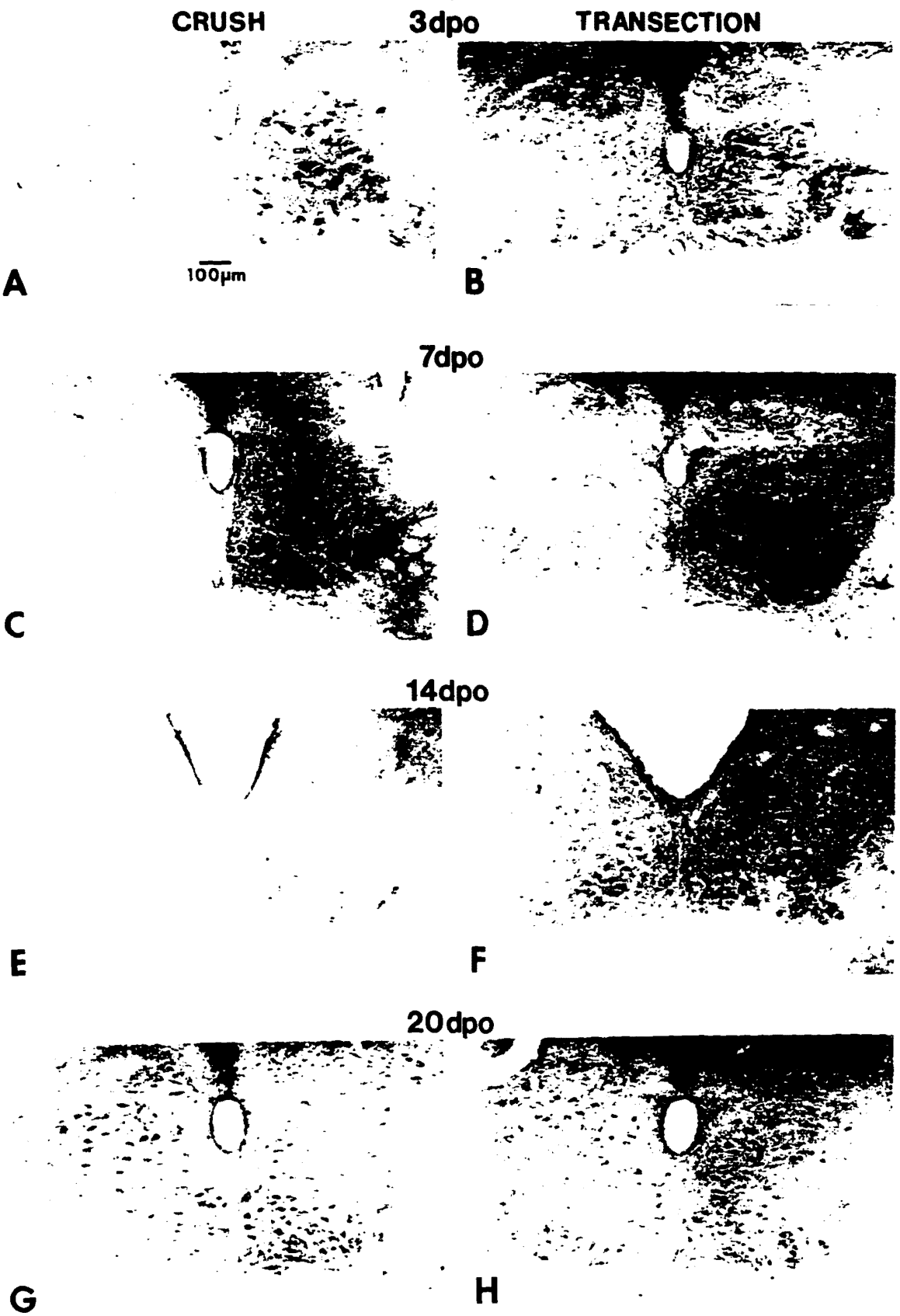


Figure. 20. LNGFR-IR in hypoglossal nuclei after unilateral hypoglossal nerve crush (left side) or transection (right side) in 21 dpn rats A-B: 3 dpo; C-D: 7 dpo; E-F: 14 dpo; G-H: 20 dpo. The injured motoneurons are located in the hypoglossal nucleus on the right side in A-H.


21 DPN-NGFr
CRUSH
3dpo
TRANSECTION
100µm
A
B
7dpo
C
D
14dpo
E
F
20dpo
G
H

elevations produced after nerve crush. In the 10 dpn nerve-transected series, LNGFR-IR was of the same intensity bilaterally in the hypoglossal nucleus on the intact and injured sides at 3 dpo as was found in 10 dpn nerve crush specimens (compare Fig. 18B to 18A). At subsequent postoperative times in the youngest rats after nerve transection, LNGFR staining persisted in the nucleus on the injured side, but little (7 dpo) to practically no (14 dpo & 20 dpo) LNGFR-IR remained in the contralateral, noninjured nucleus (Figs. 18D,F,H). In 14 dpn rats with transected axons, slight to moderate elevations of LNGFR-IR were seen in neurons on the injured side at all 4 postoperative times (3, 7, 14 & 20 dpo), but only an occasional neuron was immuno-reactive for LNGFR in the intact nucleus (Figs. 19B,D,F,H). The most marked LNGFR-IR resulted from nerve transection in 21 dpn rats. The intensity of the LNGFR-IR was elevated over that found in tissue of injured nuclei from nerve-transected rats of the other 2 ages (10 & 14 dpn), as well as more intense than staining in injured hypoglossal nuclei after nerve crush in the same age (21 dpn) rats (compare Fig. 20 to Figs. 18 & 19). Elevated levels of LNGFR-IR were maximal at 7 dpo and declined from 14-20 dpo in the 21 dpn rats (Figs. 20D,F,H).

Discussion

This work described the temporal expression of LNGFR within hypoglossal motoneurons during postnatal development and after peripheral nerve injury. The major findings of the current work were that: 1) as hypoglossal motoneurons matured postnatally LNGFR-IR disappeared; 2) denervation resulted in a reexpression of LNGFR-IR within injured hypoglossal motoneurons; 3) the magnitude of LNGFR-IR was greater after nerve crush; and 4) the denervation of LNGFR-IR was longer after transection.

Postnatal Development of LNGFR in Hypoglossal Neurons

This study examined the postnatal expression of LNGFR in hypoglossal motoneurons during postnatal development. While LNGFR is expressed in brainstem and spinal motoneurons during the prenatal period (E-13-18) (Yan & Johnson, 1988; Ernfors et al., 1989) the highest levels of LNGFR are not detected until birth and then decrease progressively to adulthood (Yan & Johnson, 1988). During postnatal development, LNGFR levels in hypoglossal motoneurons were intense during the first postnatal week, declined toward the end of the second postnatal week, and virtually disappeared by the end of the third postnatal week. The timing of the changes in LNGFR-IR postnatally correlated well

with earlier studies documenting the progression of LNGFR expression in developing brainstem and spinal motoneurons (Yan & Johnson, 1988; Ernfors et al., 1989; Chiu et al., 1993). In one study, levels of LNGFR in brainstem and spinal motoneurons peaked around birth, decreased, and virtually disappeared by the second postnatal week (Yan & Johnson, 1988).

The expression of LNGFR in motoneurons during early postnatal development is consistent with its requirement for neurotrophic support during periods of axonal growth and maturation (Ernfors et al., 1989; Rende et al., 1995). The down regulation of LNGFR postnatally is thought to be associated with completion of neurite elongation and synapse formation as the motoneuron establishes a mature connection with its target muscle (Yan & Johnson, 1988; Ernfors et al., 1989; Rende et al., 1992). Loss of LNGFR is consistent with the motoneurons' transition from target dependence to independence. This transition occurs during the middle of the first postnatal month (Lowrie & Vrbová, 1992). In the current work LNGFR disappeared as CGRP and ChAT expression reached maximal levels (discussed in Chapters 2 and 3).

Response of LNGFR to Hypoglossal Nerve Injury during Postnatal Development

While numerous studies have examined the postnatal expression of LNGFR in brainstem and spinal motoneurons of the rat (Yan & Johnson, 1988; Ernfors et al., 1989; Chiu et al. 1993), as well as the reexpression of LNGFR in adult motoneurons after nerve crush or transection (Ernfors et al., 1989; Wood et al., 1990; Armstrong et al., 1991; Koliatsos et al., 1991; Moix et al., 1991; Saika et al., 1991b; Greeson et al.,1992; Hayes et al., 1992; Rende et al.,1992; Chiu et al., 1993, Rende et al., 1993; Rende et al., 1995), no study has investigated the changes in LNGFR-IR after nerve injury to rats of increasing postnatal age.

In this work, for 10 and 14 dpn experimental rats, the intensity of the increase in LNGFR was greater after nerve crush but persisted longer after transection. These findings agree with those obtained after the same injuries to adult hypoglossal (Wood et al., 1990; Hayes et al., 1992; Rende et al., 1995) and facial (Saika et al., 1991b) motoneurons. In the 21 dpn experimental rats however, the increase in LNGFR-IR appeared more marked after nerve transection than nerve crush which has also been observed in an adult study (Armstrong et al., 1991). This difference may be related to the age. However, the fact that even within adult hypoglossal motoneuron studies there is a lack of agreement as to which type of injury triggers the stronger increase in LNGFR (compare Armstrong, et al., 1991 to Wood et al., 1990; Hayes et al., 1992; & Rende et al., 1995) suggests

that additional experiments would be needed to address whether this is a valid age-related difference.

In agreement with previous studies in the adult rat (Armstrong et al., 1991; Saika et al., 1991b; Hayes et al., 1992; Rende et al., 1995) the nerve crush response was maintained for a shorter duration postoperatively while transection resulted in a longer period of LNGFR expression. Thus, the return of LNGFR-IR was related to the type of nerve injury not the age of the injured rat. The timing of the disappearance of LNGFR may be associated with the period of axonal regrowth (Rende et al., 1992, 1993). The earlier disappearance of LNGFR-IR after nerve crush may be related to the ability of these neurons to more efficiently reinnervate their target musculature (Hayes et al., 1992). This idea is strengthened by evidence discussed in Chapter 2 indicating that motoneurons of rats at 10, 14, and 21 dpn had completely reinnervated the target by 20 dpo (21 dpn) and 40 dpo (10 and 14 dpn) following hypoglossal nerve crush. Additionally, LNGFR is expressed on Schwann cells on the distal segment of injured nerve in adult rats for a longer duration after nerve transection compared to nerve crush (Scherer et al., 1994) indicating that the signal stimulating the reexpression of LNGFR may also have a varied duration of expression at the injury site. The expression of neurotrophic factors such as NGF and BDNF are known to change in a time dependent manner following peripheral nerve injury (Heumann et al.,1987; Meyer et al., 1992; Funakoshi et al., 1993; Friedman et al., 1995a).

The induction of LNGFR following peripheral nerve injury is dependent on a signal from the injury site: LNGFR is not reexpressed if axonal transport is blocked or if the transected nerve is ligated proximal to the cut (Moix et al., 1991; Greeson et al., 1992; Hayes et al., 1992).

Neurotrophins are a family of proteins that include NGF, BDNF, NT-3, and NT-4/5 (Barde, 1989; Glass & Yancopoulos, 1993). While NGF itself has not been found to have an effect in motoneurons via LNGFR (Yan et al., 1988) there is increasing evidence that motoneurons may be responsive to other members of the neurotrophin family since BDNF, NT-3, and NT-4/5 also have the capability to bind to LNGFR (Barde, 1989; Rodriguez-Tébar et al., 1990, 1992; Glass & Yancopoulos, 1993; Yan et al., 1993). The functions of neurotrophins on different neuronal populations are mediated by the cellular expression of the trk family of tyrosine kinase receptors: Trk A is predominantly activated by NGF, Trk B by BDNF, NT-3, and NT-4/5, and Trk C by NT-3. While motoneurons fail to express the Trk A receptors they are capable of expressing Trk B and Trk C mRNAs during development (Yan et al., 1993) and into adulthood (Koliatsos et al.,1993;Yan et al., 1993). Furthermore, target muscle cells normally contain BDNF and NT-3 (Henderson et al., 1993). Nerve injury up-regulates the expression of BDNF and NT-4/5 in nonneuronal cells surrounding the distal ends of injured sciatic nerve (Meyer et al., 1992; Funakoshi et al., 1993) as well as elevates Trk B mRNA in injured motoneurons (Piehl et al., 1994). Based on recent studies demonstrating that LNGFR can function as an auxillary

molecule for Trk B it has been suggested that the increased expression of LNGFR following peripheral nerve injury may enhance the sensitivity of motoneurons to BDNF and NT-4/5 (Friedman et al., 1995b). The reexpression of LNGFR in injured postnatal motoneurons, as well as in denervated adult motoneurons may serve to concentrate large quantities of neurotrophic factors that are known to provide trophic support to injured motoneurons.

These factors may also have putative neurotrophic effects on motoneurons since labeled BDNF and NT-3 injected into the muscle (Yan et al., 1988; Yan et al. 1993) and BDNF and NT-3 injected into the crushed sciatic nerve (DiStephano et al., 1992) can be retrogradely transported. Also, local application of BDNF, NT-3, and NT-4/5 have been shown to promote motoneuron survival (Yan et al.,1992; Koliatsos et al., 1993; Wong et al., 1993; Yan et al.,1993).

Therefore, while reexpression of LNGFR is not appreciably different from that observed in adult motoneurons after the same types of nerve injury its presence strengthens the idea that it serves some role in injured motoneurons of increasing postnatal ages as well as in adult motoneurons. Further studies associated with the role of LNGFR as a receptor involved in enhanced uptake of trophic factors such as BDNF, NT-3, and NT-4/5 during times of increased trophic need such as denervation, would be necessary to further understand the mechanisms involved in the reappearance of this receptor in response to nerve injury.

CHAPTER 5.

Summary and Conclusions

Progressive changes in the expression of CGRP, ChAT, and LNGFR in normal and nerve-injured hypoglossal motoneurons during postnatal maturation in rats are summarized below.

Calcitonin Gene-Related Peptide

A. Postnatal Changes

Maximal levels of CGRP-IR were reached at 10 dpn. These levels were maintained during the first postnatal month but declined in adult motoneurons.

B. Injury Changes

1) There was an age-dependent progression in α CGRP mRNA and its peptide in injured motoneurons.
   a. α CGRP mRNA was up-regulated soon after each injury for all age groups.
   b. Subsequent elevations were limited to the 21 dpn injury paradigms.

2) If the nerve injury occurs at a postnatal age (21 dpn) when the CGRP response parallels that of adult motoneurons, neuronal survival and reinnervation were maximized.

Choline Acetyltranseferase

A. Postnatal Changes

Maximal levels of ChAT-IR were reached at 10 dpn and the intensity of enzyme became conspicuous in the neuropil from 17-30 dpn.

B. Injury Changes

1) The changes in ChAT-IR after nerve crush or transection in the 10, 14, and 21 dpn rats paralleled those reported after the same injuries to adult motoneurons.

2) The timing of the onset of ChAT-IR was not age-related or injury-related. However, the magnitude of the initial reduction of ChAT was injury-specific for the 21 dpn rats but not for the 10 and 14 dpn rats.

3) In all injury paradigms, ChAT returned to normal levels by 40 dpo except in the 10 dpn rats nerve transected rats.

Low-Affinity Nerve Growth Factor

A. Postnatal Changes

Intense levels of LNGFR-IR in the first postnatal week, gradually decreased in the second postnatal week and disappeared by 21 dpn.

B. Injury Changes

Axonal injury resulted in a re-expression of LNGFR-IR within injured hypoglossal motoneurons.

a. The re-expression of LNGFR-IR was not age-related after nerve injury in the 14 and 21 dpn rats.

b. The magnitude and duration of LNGFR-IR were injury specific.

On the basis of these findings, the final conclusions were made.

1. The onset of changes in ChAT, LNGFR, and CGRP is the same after axonal damage to motoneurons in postnatal and adult rats.

2. After onset, the progression of changes in ChAT and LNGFR is not age-related but injury-specific as reported for adult motoneurons.

3. The progression of changes in CGRP after onset is age-related and likely to depend upon the developmental stage of motoneuron interaction with its target and/or its environment.

4. If injury occurs at a postnatal age when the immunoreactive response of CGRP parallels that of adult motoneurons, neuronal survival can be predicted.

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